TITLE: METHOD FOR MODIFYING PLANT MORPHOLOGY, BIOCHEMISTRY AND PHYSIOLOGY

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DOCKET: 57955/010

METHOD FOR MODIFYING PLANT MORPHOLOGY, BIOCHEMISTRY AND PHYSIOLOGY

This application is a continuation-in-part application of PCT/EP01/06833, having an international filing date of June 18, 2001.

FIELD OF THE INVENTION

The present invention generally relates to methods for modifying plant morphological, biochemical and physiological properties or characteristics, such as one or more developmental processes and/or environmental adaptive processes, including but not limited to the modification of initiation or stimulation or enhancement of root growth, and/or adventitious root formation, and/or lateral root formation, and/or root geotropism, and/or shoot growth, and/or apical dominance, and/or branching, and/or timing of senescence, and/or timing of flowering, and/or flower formation, and/or seed development, and/or seed yield. Methods for increasing seed size and/or weight, increasing embryo size and/or weight, and increasing cotyledon size and/or weight are also provided. The methods comprise expressing a cytokinin degradation control protein, in particular cytokinin oxidase, in the plant, operably under the control of a regulatable promoter sequence such as a cell-specific promoter, tissue-specific promoter, or organ-specific promoter sequence. Preferably, the characteristics modified by the present invention are cytokinin-mediated and/or auxin-mediated characteristics. The present invention extends to genetic constructs which are useful for performing the inventive method and to transgenic plants produced therewith having altered morphological and/or biochemical and/or physiological properties compared to their otherwise isogenic counterparts.

BACKGROUND OF THE INVENTION

Roots are an important organ of higher plants. Their main functions are anchoring of the plant in the soil and uptake of water and nutrients (N-nutrition, minerals, etc.). Thus, root growth has a direct or indirect influence on growth and yield of aerial organs, particularly under conditions of nutrient limitation. Roots are also relevant for the production of secondary plant products, such as defense compounds and plant hormones.

Roots are also storage organs in a number of important staple crops. Sugar beet is the most important plant for sugar production in Europe (260 Mill t/year; 38 % of world production). Manioc (cassava), yams and sweet potato (batate) are important starch producers (app. 150 Mill t/year each). Their content in starch can be twice as high as that of potato. Roots are also the relevant organ for consumption in a number of vegetables (e.g. carrots, radish), herbs (e.g. ginger, kukuma) and medicinal plants (e.g. ginseng). In addition, some of the secondary plant products found in roots are of economic importance for the chemical and pharmaceutical industry. An example is yams, which contain basic molecules for the synthesis of steroid hormones. Another example is shikonin, which is produced by the roots of *Lithospermum erythrorhizon* in hairy root cultures. Shikonin is used for its anti-inflammatory, anti-tumor and wound-healing properties.

Moreover, improved root growth of crop plants will also enhance competitiveness with weedy plants and will improve growth in arid areas, by increasing water accessibility and uptake.

Improved root growth is also relevant for ecological purposes, such as bioremediation and prevention/arrest of soil erosion.

Root architecture is an area that has remained largely unexplored through classical breeding, because of difficulties with assessing this trait in the field. Thus, biotechnology could have significant impact on the improvement of this trait, because it does not rely on large-scale screenings in the field. Rather, biotechnological approaches require a basic understanding of the molecular components that determine a specific characteristic of the plant. Today, this knowledge is only fragmentary, and as a consequence, biotechnology was so far unable to realize a break-through in this area.

A well-established regulator of root growth is auxin. Application of indole-3-acetic acid (IAA) to growing plants stimulates lateral root development and lateral root elongation (Torrey, Am J Bot 37: 257-264, 1950; Blakely *et al.*, Bot Gaz 143: 341-352, 1982; Muday and Haworth, Plant Physiol Biochem 32:

193-203, 1994). Roots exposed to a range of concentrations of IAA initiated increasing numbers of lateral roots (Kerk *et al.*, Plant Physiol, 122: 925-932, 2000). Furthermore, when roots that had produced laterals in response to a particular concentration of exogenous auxin were subsequently exposed to a higher concentration of IAA, numerous supernumerary lateral roots spaced between existing ones were formed (Kerk *et al.*, Plant Physiol, 122: 925-932, 2000). Conversely, growth of roots on agar containing auxin-transport inhibitors, including NPA, decreases the number of lateral roots (Muday and Haworth, Plant Physiol Biochem 32: 193-203, 1994).

Arabidopsis mutants containing increased levels of endogenous IAA have been isolated (Boerjan et al., Plant Cell 7: 1405-141, 1995; Celenza et al., Gene Dev 9: 2131-2142, 1995; King et al., Plant Cell 7: 2023-2037, 1995; Lehman et al., Cell 85: 183-194, 1996). They are now known to be alleles of a single locus located on chromosome 2. These mutant seedlings have excess adventitious and lateral roots, which is in accordance with the above-described effects of external auxin application.

The stimulatory effect of auxins on adventitious and lateral root formation suggests that overproduction of auxins in transgenic plants is a valid strategy for increasing root growth. Yet, it is also questionable whether this would yield a commercial product with improved characteristics. Apart from its stimulatory effect on adventitious and lateral root formation, auxin overproduction triggers other effects, such as reduction in leaf number, abnormal leaf morphology (narrow, curled leaves), aborted inflorescences, increased apical dominance, adventitious root formation on the stem, most of which are undesirable from an agronomic perspective (Klee *et al.*, Genes Devel 1: 86-96, 1987; Kares *et al.*, Plant Mol Biol 15: 225-236, 1990). Therefore, the major problem with approaches that rely on increased auxin synthesis is a problem of containment, namely to confine the effects of auxin to the root. This problem of containment is not likely overcome by using tissue-specific promoters: auxins are transported in the plant and their action is consequently not confined to the site of synthesis. Another issue is whether auxins will always enhance the total root biomass. For agar-grown plants, it has been noticed that increasing concentrations progressively

stimulated lateral root formation but concurrently inhibited the outgrowth of these roots (Kerk *et al.*, Plant Physiol, 122: 925-932, 2000).

Seeds are the reproduction unit of higher plants. Plant seeds contain reserve compounds to ensure nutrition of the embryo after germination. These storage organs contribute significantly to human nutrition as well as cattle feeding. Seeds consist of three major parts, namely the embryo, the endosperm and the seed coat. Reserve compounds are deposited in the storage organ which is either the endosperm (resulting form double fertilisation; e.g. in all cereals), the so-called perisperm (derived from the nucellus tissue) or the cotyledons (e.g. bean varieties). Storage compounds are lipids (oil seed rape), proteins (e.g. in the aleuron of cereals) or carbohydrates (starch, oligosaccharides like raffinose).

Starch is the storage compound in the seeds of cereals. The most important species are maize (yearly production ca. 570 mio t; according to FAO 1995), rice (540 mio t p.a.) and wheat (530 mio t p.a.). Protein rich seeds are different kinds of beans (*Phaseolus spec., Vicia faba*, *Vigna spec.*; ca. 20 mio t p.a.), pea (*Pisum sativum;* 14 mio t p.a.) and soybean (*Glycine max;* 136 mio t p.a.). Soybean seeds are also an important source of lipids. Lipid rich seeds are as well those of different *Brassica* species (app. 30 mio t p.a.), cotton, oriental sesame, flax, poppy, castor bean, sunflower, peanut, coconut, oilpalm and some other plants of less economic importance.

After fertilization, the developing seed becomes a sink organ that attracts nutritional compounds from source organs of the plant and uses them to produce the reserve compounds in the storage organ. Increases in seed size and weight, are desirable for many different crop species. In addition to increased starch, protein and lipid reserves and hence enhanced nutrition upon ingestion, increases in seed size and/or weight and cotyledon size and/or weight are correlated with faster growth upon germination (early vigor) and enhanced stress tolerance. Cytokinins are an important factor in determining sink strength. The common concept predicts that cytokinins are a positive regulator of sink strength.

Numerous reports ascribe a stimulatory or inhibitory function to cytokinins in different developmental processes such as root growth and branching, control of apical dominance in the shoot, chloroplast development, and leaf senescence (Mok M.C. (1994) in *Cytokines: Chemistry, Activity and Function,* eds., Mok, D.W.S. & Mok,M.C. (CRC Boca Raton, Fl), pp.155-166). Conclusions about the biological functions of cytokinins have mainly been derived from studies on the consequences of exogenous cytokinin application or endogenously enhanced cytokinin levels (Klee, H.J. & Lanehon, M.B. (1995) in *Plant Hormones:Physiology, Biochemisry and Molecular Biology*, ed. Davies, P.J. (Kluwer, Dordrdrocht, the Netherlands), pp. 340-353, Smulling, T., Rupp, H.M. Frank, M& Schafer, S. (1999) in *Advances in Regulation of Plant Growth and Development*, eds. Surnad, M. Pac P. & Beck, E. (Peres, Prague), pp. 85-96). Up to now, it has not been possible to address the reverse question: what are the consequences for plant growth and development if the endogenous cytokinin concentration is decreased? Plants with a reduced cytokinin content are expected to yield more precise information about processes cytokinins limit and, therefore, might regulate. Unlike other plant hormones such as abscisic acid, gibberellins, and ethylene, no cytokinin biosynthetic mutants have been isolated (Hooykens, P.J.J.,Hall, M.A. & Libbeuga, K.R.,eds.(1999) *Biochemistry and Molecular Biology of Plant Hormones* (Elsevier, Amsterdam).

The catabolic enzyme cytokinin oxidase (CKX) plays a principal role in controlling cytokinin levels in plant tissues. CKX activity has been found in a great number of higher plants and in different plant tissues. The enzyme is a FAD-containing oxidoreductase that catalyzes the degradation of cytokinins bearing unsaturated isoprenoid side chains. The free bases iP and Z, and their respective ribosides are the preferred substrates. The reaction products of iP catabolism are adenine and the unsaturated aldehyde 3-methyl-2-butonal (Armstrong, D.J. (1994) in *Cytokinins: Chemistry, Activity and Functions*, eds. Mok. D.W.S & Mok, M.C. (CRC Boca Raton, FL), pp. 139-154). Recently, a cytokinin oxidase gene from *Zea mays* has been isolated (Morris, R.O., Bilyeu, K.D., Laskey, J.G. & Cherich, N.N. (1999) *Biochem. Biophys.Res. Commun. 255,* 328-333, Houba-Heria, N.,Pethe, C. d'Alayer, J & Lelouc, M. (1999) *Plant J.*

*17:*615-626). The manipulation of CKX gene expression could partially overcome the lack of cytokinin biosynthetic mutants and can be used as a powerful tool to study the relevance of iP – and Z-type cytokinins during the whole life cycle of higher plants.

The present invention overcomes problems related to containment of auxin effects, maintenance of root outgrowth, and promotion of increased seed, embryo, and cotyledon size and/or weight through reduction of endogenous cytokinin concentration.

SUMMARY OF THE INVENTION

The present invention provides plant cytokinin oxidase proteins, nucleic acid sequences encoding such proteins, and vectors, host cells and transgenic plant cells, plants, and plant parts comprising the proteins, nucleic acid sequences, and vectors. For example, the present invention relates to a genetic construct comprising a gene encoding a protein with cytokinin oxidase activity from *Arabidopsis thaliana*. This gene may be expressed under control of a regulated promoter. This promoter may be regulated by endogenous tissue-specific or environment-specific factors or, alternatively, it may be induced by application of specific chemicals.

The present invention also relates to a method to modify root architecture and biomass by expression of a cytokinin oxidase gene or expression of a nucleic acid encoding a protein that reduces the level of active cytokinins in plants or plant parts. Preferably, expression is under control of a promoter that is specific to the root or to certain tissues or cell types of the root.

Additionally, the present invention relates to methods of increasing seed size and/or weight, embryo size and/or weight, and cotyledon size and/or weight. The methods involve expression of a cytokinin oxidase gene or expression of a nucleic acid encoding a protein that reduces the level of active cytokinins in plants or plant parts. Preferably, expression is under control of a promoter directs expression preferentially in the seed, embryo, or cotyledon.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic representation of plant cytokinin oxidase genes. Shown are the structures of different cytokinin oxidase genes isolated from maize (*ZmCKX1*, accession number AF044603, Biochem. Biophys. Res. Com. 255:328-333, 1999) and Arabidopsis (*AtCKX1 to AtCKX4*). Exons are denominated with 'E' and represented by shaded boxes. Introns are represented by white boxes. Further indicated are the gene sizes (in kb, on top of each structure), the gene accession numbers (under the names) and a size bar representing 0.5 kb.

Figure 2. Alignment of plant cytokinin oxidase amino acid sequences. The amino acid sequences from cytokinin oxidases from maize (ZmCKX1) and Arabidopsis (AtCKX1 to AtCKX4) are aligned. Identical amino acid residues are marked by a black box, similar amino acid residues are in a grey box. Amino acid similarity groups: (M,I,L,V), (F,W,Y), (G,A), (S,T), (R,K,H), (E,D), (N,Q),

Figure 3. Northern blot analysis of *AtCKX1*-expressing tobacco and *Arabidopsis* plants.
(A) Northern blot analysis of constitutively expressing tobacco plants (lanes 1-8) compared to wild type SNN tobacco (lane 9)

(B) Comparison of tetracycline-induced gene expression in leaves after 12h of induction with a constitutively expressing clone. Lanes 2-9, leaves of four different *AtCKX1*-W38TetR clones (+,-, with or without tetracycline treatment), lane 1, constitutively expressing *35S:: AtCKX1* clone.

(C) Northern blot analysis of *Arabidopsis* plants constitutively expressing *AtCKX1* gene. Lanes 2-4, three different constitutively expressing *35S::AtCKX1* clones compared to wild type Arabidopsis plant (lane 1).

Figure 4: Growth characteristics of *35S::AtCKX1* transgenic *Arabidopsis* plants.

(A) Two wild type seedlings (left) compared to two *35S::AtCKX1* expressing seedlings (right). Note the increased formation of adventitious roots and increased root branching in the transgenic seedlings. Pictures were taken 14 days after germination. Plants were grown *in vitro* on MS medium in petri dishes in a vertical position.

(B) Like A, but roots stained with toluidine blue.

(C) Top view of a petri dish with *35S::AtCKX1* transgenic seedlings three weeks after germination.

(D) A *35S::AtCKX1* transgenic plants grown in liquid culture. Roots of wild type seedlings grow poorly under these conditions (not shown).

(E) Transformants (T0) that express the *35S::AtCKX1* gene (three plants on the right), a wild type plant is shown on the left.

(F) Phenotype of T1 plants grown in soil. Wild type plant (left) compared to two *35S::AtCKX1* transgenic plants.

Figure 5: Phenotype of *AtCKX2* overexpressing Arabidopsis plants.

T1 generation of *35S::AtCKX2* expressing Arabidopsis plants (two plants on the right) compared to wild type (plant on the left).

Figure 6. Northern blot analysis of *AtCKX2*-expressing tobacco and Arabidopsis plants.

(A) Northern blot analysis of constitutively expressing tobacco plants (lanes 1-7) compared to wild type SNN tobacco (lane 8)

(B) Northern blot analysis of Arabidopsis plants constitutively expressing *AtCKX2* gene. Lanes 2-8, seven different constitutively expressing *35S::AtCKX2* clones compared to wild type Arabidopsis plant (lane 1).

Figure 7. Shoot phenotype of *AtCKX1* and *AtCKX2* expressing tobacco plants.

(A) Top view of six week old plants.

(B) Tobacco plants at the flowering stage.

(C) Kinetics of stem elongation. Arrows mark the onset of flowering. Age of plants (days after germination) and leaf number at that stage are indicated above the arrows. Bars indicate SD; $n = 12$.

(D) Number of leaves ($n = 12$) formed between day 68 and day 100 after germination and final surface area of these leaves (100% of wild type is 3646 $\pm 144\ cm^2$; $n = 3$).

(E) Comparison of leaf size and senescence. Leaves were from nodes number 4, 9, 12, 16 and 20 from the top (from left to right).

Figure 8. Root phenotype of *AtCKX* expressing transgenic tobacco plants.

(A) Seedlings 17 days after germination.

(B) Root system of soil grown plants at the flowering stage.

(C) Root length, number of lateral roots (LR) and adventitious roots (AR) on day 10 after germination.

(D) Dose-response curve of root growth inhibition by exogenous cytokinin. Bars indicate ±SD; $n = 30$.

Figure 9: Growth of axillary shoot meristems in *35S::AtCKX1* expressing tobacco plants.

Figure 10: Histology of shoot meristems, leaves and root meristems of *AtCKX1* overexpressing tobacco plants versus wild type (WT) tobacco.

(A) Longitudinal median section through the vegetative shoot apical meristem. P, leaf primordia.

(B) Vascular tissue in second order veins of leaves. X, xylem, PH, a phloem bundle.

(C) Cross sections of fully developed leaves.

(D) Scanning electron microscopy of the upper leaf epidermis.

(E) Root apices stained with DAPI. RM, root meristem.

(F) Longitudinal median sections of root meristems ten days after germination. RC, root cap; PM, promeristem.

(G) Transverse root sections 10 mm from the apex. E, epidermis, C1-C4, cortical cell layer, X, xylem, PH, phloem. Bars are 100 μm.

Figure 11: Northern blot analysis of *AtCKX3* and *AtCKX4*-expressing tobacco plants.

(A) Northern blot analysis of constitutively expressing *AtCKX3* tobacco plants. Lane designations indicate individual transgenic plant numbers, WT is wild type SNN tobacco. The blot on top was probed with a *AtCKX3* specific probe, the lower blot with a probe specific for the 25S rRNA and serves as a control for RNA loading.

(B) Northern blot analysis of constitutively expressing *AtCKX4* tobacco plants. Lane designations indicate individual transgenic plant numbers, WT is wild type SNN tobacco. The blot on top was probed with an *AtCKX4* specific probe, the lower blot with a probe specific for the 25S rRNA and serves as a control for RNA loading.

Figure 12: Reciprocal grafts of *AtCKX2* transgenic tobacco plants and wild type plants.

(A) Two plants on the left: Control (WT scion grafted on a WT rootstock).

Two plants on the right: WT scion grafted on a *AtCKX2-38* transgenic rootstock.

(B) Left: Control (WT scion grafted on a WT rootstock).

Right: Scion of *AtCKX2-38* plant grafted on WT rootstock.

(C) Magnification of root area.

Left: Control (WT scion grafted on a WT rootstock).

Right: WT scion grafted on an *AtCKX2-38* transgenic rootstock.

(D) Formation of adventitious roots.

Left: Control (WT scion grafted on an WT rootstock).

Right: WT scion grafted on an *AtCKX2-38* transgenic rootstock.

Figure 13: Phenotype of *Arabidopsis* seeds, embryos and seedlings.

(A) Seeds of an *AtCKX1* transgenic line and wild type seeds. Bar size 1mm.

(B) Seeds of *AtCKX1*, *AtCKX2*, *AtCKX3* and *AtCKX4* transgenic lines and wild type seeds. Bar size 1 mm.

(C) Mature embryos of *AtCKX1* transgenic *Arabidopsis* and of a wild type plant. Bar size 200 µm. Embryos were obtained from mature seeds that had been imbibed for 12 hours in 20% EtOH, squeezed out from the seed coat, cleared with chloralhydrate and photographed using Nomarski optics.

(D) Wild type (top) and *AtCKX1* expressing *Arabidopsis* seedlings 4 days after germination.

(E) Close-up of D.

Figure 14: Seed weight of wild type and two independent clones for each of the four investigated AtCKX genes. Average weight obtained by analysing five different batches of 200 seeds for each clone.

DETAILED DESCRIPTION OF THE INVENTION

To by-pass above-mentioned problems associated with increasing auxin biosynthesis, it was decided to follow an alternative approach. We reasoned that down-regulation of biological antagonists of auxins could evoke similar or even superior effects on root growth as compared to increasing auxin levels. Hormone actions and interactions are extremely complex, but we hypothesized that cytokinins could function as auxin antagonists with respect to root growth. Hormone studies on plant tissue cultures have shown that the ratio of auxin versus cytokinin is more important for organogenesis than the absolute levels of each of these hormones, which indeed indicates that these hormones function as antagonists – at least in certain biological processes. Furthermore, lateral root formation is inhibited by exogenous application of cytokinins. Interestingly, also root elongation is negatively affected by cytokinin treatment, which suggests that cytokinins control both root branching and root outgrowth.

Together, current literature data indicate that increasing cytokinin levels negatively affects root growth, but the mechanisms underlying this process are not understood. The sites of cytokinin synthesis in the plant are root tips and young tissues of the shoot. Endogenous concentrations of cytokinins are in the nM range. However, as their quantification is difficult, rather large tissue amounts need to be extracted and actual local concentrations are not known. Also the subcellular compartmentation of cytokinins is not known. It is generally thought that the free base and ribosides are localized in the cytoplasm and nucleus, while glucosides are localized in the vacuole. There exist also different cytokinins with slightly different chemical structure. As a consequence, it is not known whether the effects of exogenous cytokinins should be ascribed to a raise in total cytokinin concentration or rather to the competing out of other forms of plant-borne cytokinins (which differ either in structure, cellular or subcellular location) for receptors, translocators, transporters, and modifying enzymes.

In order to test the hypothesis that cytokinin levels in the root indeed exceed the level optimal for root growth, novel genes encoding cytokinin oxidases (which are cytokinin metabolizing enzymes) were cloned from *Arabidopsis thaliana* (designated *AtCKX*) and were subsequently expressed under a strong constitutive promoter in transgenic tobacco and *Arabidopsis*. Transformants showing *AtCKX* mRNA expression and increased cytokinin oxidase activity also manifested enhanced formation and growth of roots. Negative effects on shoot growth were also observed. The latter is in accordance with the constitutive expression of the cytokinin oxidase gene in these plants, illustrating the importance of confined expression of the cytokinin oxidase gene for general plant growth properties. Containment of cytokinin oxidase activity can be achieved by using cell-, tissue- or organ-specific promoters, since cytokinin degradation is a process limited to the tissues or cells that express the CKX protein, this in contrast to approaches relying on hormone synthesis, as explained above.

The observed negative effects of cytokinin oxidase expression on shoot growth demonstrate that cytokinin oxidases are interesting targets for the design of or screening for growth-promoting chemicals. Such chemicals should inhibit cytokinin oxidase activity, should preferably not be transported to the root and should be rapidly degraded in soil, so that application of these chemicals will not inhibit root growth. Cytokinins also delay leaf senescence, which means that positive effects will include both growth and maintenance of photosynthetic tissues. In addition, the observation that cytokinins delay senescence, enhance greening (chlorophyll content) of leaves and reduce shoot apical dominance shows that strategies based on suppressing CKX activity (such as antisense, ribozyme, and cosuppression technology) in the aerial parts of the plant could result in delayed senescence, enhanced leaf greening and increased branching.

Similarly, the observed positive effects of cytokinin oxidase expression on root growth demonstrate that cytokinin oxidases are interesting targets for the design of or screening for herbicides. Such herbicides should inhibit cytokinin oxidase activity, should preferably not be transported to the shoot, and should be soluble and relatively stable in a solvent that can be administered to the root through the soil.

These effects of cytokinin oxidase overexpression on plant development and architecture were hitherto unknown and, as a consequence, the presented invention and its embodiments could not be envisaged.

The observed negative effects on shoot growth demonstrate that manipulation of cytokinin oxidases can also be used for obtaining dwarfing phenotypes. Dwarfing phenotypes are particularly useful in commercial crops such as cereals and fruit trees for example.

In accordance with the present invention, it has also been surprisingly discovered that transgenic plants overexpressing a cytokinin oxidase gene develop seeds (including embryos) and cotyledons of increased size and/or weight. These results are surprising as a reduced cytokinin content would have been expected to be associated with a reduced organ growth.

Preferable embodiments of the invention relate to the positive effect of cytokinin oxidase expression on plant growth and architecture, and in particular on root growth and architecture, seed size and weight, embryo size and weight, and cotyledon size and weight. The cytokinin oxidase gene family contains at least six members in *Arabidopsis* (see examples below) and the present inventors have shown that there are quantitative differences in the effects achieved with some of these genes in transgenic plants. It is anticipated that functional homologs of the described *Arabidopsis* cytokinin oxidases can be isolated from other organisms, given the evidence for the presence of cytokinin oxidase activity in many green plants (Hare and van Staden, Physiol Plant 91:128-136, 1994; Jones and Schreiber, Plant Growth Reg 23:123-134, 1997), as well as in other organisms (Armstrong, in Cytokinins: Chemistry, Activity and Function. Eds Mok and Mok, CRC Press, pp139-154, 1994). Therefore, the sequence of the cytokinin oxidase, functional in the invention, need not to be identical to those described herein. This invention is particularly useful for cereal crops and monocot crops in general and cytokinin oxidase genes from for example wheat or maize may be used as well (Morris et al., 1999; Rinaldi and Comandini, 1999). It is envisaged that other genes with cytokinin oxidase activity or with any other cytokinin metabolizing activity (see Zažímalová *et al.*, Biochemistry and Molecular Biology

of Plant Hormones, Hooykaas, Hall and Libbenga (Eds.), Elsevier Science, pp141-160, 1997) can also be used for the purpose of this invention. Similarly, genes encoding proteins that would increase endogenous cytokinin metabolizing activity can also be used for the purpose of this invention. In principle, similar phenotypes could also be obtained by interfering with genes that function downstream of cytokinin such as receptors or proteins involved in signal transduction pathways of cytokinin.

For the purpose of this invention, it should be understood that the term 'root growth' encompasses all aspects of growth of the different parts that make up the root system at different stages of its development, both in monocotyledonous and dicotyledonous plants. It is to be understood that enhanced growth of the root can result from enhanced growth of one or more of its parts including the primary root, lateral roots, adventitious roots, etc. all of which fall within the scope of this invention.

For purposes of this invention, it should also be understood that increases in seed weight or seed size can include increases in the size of one or more of the embryo, the endosperm, aleurone, and seed coat. Moreover, increases in embryo size and/or weight can include increases in different organs associated therewith such as e.g., cotyledons, hypocotyl, and roots.

According to a first embodiment, the present invention relates to a method for stimulating root growth and/or enhancing the formation of lateral and/or adventitious roots and/or altering root geotropism comprising expression of a plant cytokinin oxidase or comprising expression of another protein that reduces the level of active cytokinins in plants or plant parts.

In another embodiment, the present invention relates to a method for increasing plant seed size and/or weight, by increasing the level or activity of a cytokinin oxidase in the plant or by expression of another protein that reduces the level of active cytokinins in a plant or plant part. Preferably, the increased level or activity of a cytokinin oxidase or expression of another protein that reduces the

level of active cytokinins in a plant or plant part is localized in the seed including different tissues or cell types of the seed.

In another embodiment, the present invention relates to a method for increasing plant embryo size and/or weight, by increasing the level or activity of a cytokinin oxidase in the plant or by expression of another protein that reduces the level of active cytokinins in a plant or plant part. Preferably, the increased level or activity of a cytokinin oxidase or expression of another protein that reduces the level of active cytokinins in a plant or plant part is localized in the seed. Even more preferably, the increased level or activity of a cytokinin oxidase or expression of another protein that reduces the level of active cytokinins in a plant or plant part is localized in the embryo.

In yet another embodiment, the present invention relates to a method for increasing plant cotyledon size and/or weight, by increasing the level or activity of a cytokinin oxidase in the plant or by expression of another protein that reduces the level of active cytokinins in a plant or plant part. Preferably, the increased level or activity of a cytokinin oxidase or expression of another protein that reduces the level of active cytokinins in a plant or plant part is localized in the cotyledon.

In the context of the present invention it should be understood that the term "expression" and/or 'overexpression' are used interchangeably and both relate to an "enhanced and/or ectopic expression" of a plant cytokinin oxidase or any other protein that reduces the level of active cytokinins in plants. It should be clear that herewith an enhanced expression of the plant cytokinin oxidase as well as "de novo" expression of plant cytokinin oxidases or of said other proteins is meant. Alternatively, said other protein enhances the cytokinin metabolizing activity of a plant cytokinin oxidase.

It further should be understood that in the context of the present invention the expression "lateral and/or adventitious roots" can mean "lateral and adventitious roots" but also "lateral or adventitious roots". The enhancement can

exist in the formation of lateral roots or in the formation of adventitious roots as well as in the formation of both types of non-primary roots, but not necessarily.

In addition, as used herein, "increasing seed size and/or weight," can mean increasing seed size and weight, but also size or weight. Thus, the enhancement can exist in an increase in the size of the seed or the weight of the seed or both. Similar interpretations should be applied to "increasing embryo size and/or weight" and "increasing cotyledon size and/or weight."

The terms "plant" and "plant part" are used interchangeably with the terms "plants" and "plant parts."

According to a further embodiment, the present invention relates to a method for stimulating root growth and/or enhancing the formation of lateral or adventitious roots and/or altering root geotropism and/or increasing yield and/or enhancing early vigor and/or modifying root/shoot ratio and/or improving resistance to lodging and/or increasing drought tolerance and/or promoting in vitro propagation of explants, comprising expression of a plant cytokinin oxidase or comprising expression of another protein that reduces the level of active cytokinins in plants or plant parts.

According to a preferred embodiment, the present invention relates to a method for stimulating root growth resulting in an increase of root mass by overexpression of a cytokinin oxidase, preferably a cytokinin oxidase according to the invention, or another protein that reduces the level of active cytokinins in plants or plant parts, preferably in roots.

Higher root biomass production due to overexpression of growth promoting sequences has a direct effect on the yield and an indirect effect of production of compounds produced by root cells or transgenic root cells or cell cultures of said transgenic root cells. One example of an interesting compound produced in root cultures is shikonin, the yield of which can be advantageously enhanced by said methods.

According to a more specific embodiment, the present invention relates to methods for stimulating root growth or for enhancing the formation of lateral and/or adventitious roots or for altering root geotropism or for increasing seed size and/or weight, or for increasing embryo size and/or weight, or for increasing cotyledon size and/or weight. The methods comprise expression of a nucleic acid encoding a plant cytokinin oxidase selected from the group consisting of:

(a) nucleic acids comprising a DNA sequence as given in any of SEQ ID NOs: 27, 1, 3, 5, 7, 9, 11, 25, 26, 28 to 31, 33 or 34, or the complement thereof,

(b) nucleic acids comprising the RNA sequences corresponding to any of SEQ ID NOs: 27, 1, 3, 5, 7, 9, 11, 25, 26, 28 to 31, 33 or 34, or the complement thereof,

(c) nucleic acids specifically hybridizing to any of SEQ ID NOs: 27, 1, 3, 5, 7, 9, 11, 25, 26, 28 to 31, 33 or 34, or to the complement thereof,

(d) nucleic acids encoding a protein comprising the amino acid sequence as given in any of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 32 or 35, or the complement thereof,

(e) nucleic acids as defined in any of (a) to (d) characterized in that said nucleic acid is DNA, genomic DNA, cDNA, synthetic DNA or RNA wherein T is replaced by U,

(f) nucleic acids which are degenerated to a nucleic acid as given in any of SEQ ID NOs: 27, 1, 3, 5, 7, 9, 11, 25, 26, 28 to 31, 33 or 34, or which are degenerated to a nucleic acid as defined in any of (a) to (e) as a result of the genetic code,

(g) nucleic acids which are diverging from a nucleic acid encoding a protein as given in any of SEQ ID NOs: 2, 4, 6, 8, 10, 12 or 35 or which are diverging from a nucleic acid as defined in any of (a) to (e), due to the differences in codon usage between the organisms,

(h) nucleic acids encoding a protein as given in SEQ ID NOs: 2, 4, 6, 8, 10, 12 or 35 or nucleic acids as defined in (a) to (e) which are diverging due to the differences between alleles,

(i) nucleic acids encoding a protein as given in any of SEQ ID NOs: 2, 4, 6, 8, 10, 12 or 35,

(j) functional fragments of nucleic acids as defined in any of (a) to (i) having the biological activity of a cytokinin oxidase, and

(k) nucleic acids encoding a plant cytokinin oxidase,

or comprise expression, preferably in roots, or in seeds (including parts of seeds such as embryo, endosperm, seed coat or aleurone) or in cotyledons, of a nucleic acid encoding a protein that reduces the level of active cytokinins in plants or plant parts.

In the present invention, nucleic acids encoding novel *Arabidopsis thaliana* cytokinin oxidases have been isolated and for the first time, the present inventors have surprisingly shown that the expression of cytokinin oxidases in transgenic plants or in transgenic plant parts resulted in the above-mentioned root and seed-related features. In order that root-related features be effected, the expression of the cytokinin oxidase(s) should take place in roots, preferably under the control of a root-specific promoter. In order that seed-related features be effected (including the embryo), expression of the cytokinin oxidase(s) should take place in seeds, preferably under the control of a seed-specific promoter. One example of such a root-specific promoter is provided in SEQ ID NO: 36. Examples of seed-specific promoters include but are not limited to those listed in Table 4.

In order that cotyledon-related features be effected, the expression of the cytokinin oxidase(s) should take place in the cotyledons, preferably under the control of a promoter which preferentially expresses in cotyledon.

It should be clear that, although the invention is supported in the examples section by several new AtCKX genes and proteins, the inventive concept also

relates to the use of other cytokinin oxidases isolated from and expressed in other plants, preferably in the roots and/or seeds and/or cotyledons of said other plants to obtain similar effects in plants as described in the examples section.

Therefore, the present invention more generally relates to the use of a nucleic acid encoding a plant cytokinin oxidase or encoding a protein that reduces the level of active cytokinins in plants or plant parts for stimulating root growth or for enhancing the formation of lateral or adventitious roots or for altering root geotropism. The present invention also relates to the use of a nucleic acid encoding a plant cytokinin oxidase or encoding a protein that reduces the level of active cytokinins in plants or plant parts for increasing seed size and/or weight, or for increasing embryo size and/or weight, or for increasing plant cotyledon size and/or weight. Preferred cytokinin oxidases to be used are encoded by the nucleic acids encoding the cytokinin oxidases as defined above and are encoded by the novel nucleic acids of the invention as defined hereunder.

The invention relates to an isolated nucleic acid encoding a novel plant protein having cytokinin oxidase activity selected from the group consisting of:

(a) a nucleic acid comprising a DNA sequence as given in any of SEQ ID NOs: 29, 3, 5, 9, 26, 27, 31, 33 or 34, or the complement thereof,

(b) a nucleic acid comprising the RNA sequences corresponding to any of SEQ ID NOs: 29, 3, 5, 9, 26, 27, 31, 33 or 34, or the complement thereof,

(c) a nucleic acid specifically hybridizing to a nucleic acid as given in any of SEQ ID NOs: 29, 3, 5, 9, 26, 27, 31, 33 or 34, or the complement thereof,

(d) a nucleic acid encoding a protein with an amino acid sequence comprising the polypeptide as given in SEQ ID NO: 32 and which is at least 70% similar, preferably at least 75%, 80% or 85%, more preferably at least 90% or 95%, most preferably at least 99% similar to the amino acid sequence as given in SEQ ID NO: 4,

(e) a nucleic acid encoding a protein with an amino acid sequence which is at least 35% similar, preferably 37%, 40%, 45%, 47% or 50%, similar,

more preferably 55%, 60%, 65%, 70%, 75% or 80% similar, most preferably 85%, 90% or 95% similar to the amino acid sequence as given in SEQ ID NO: 6,

(f) a nucleic acid encoding a protein with an amino acid sequence which is at least 35% similar, preferably 37%, 40%, 45%, 47% or 50%, similar, more preferably 55%, 60%, 65%, 70%, 75% or 80% similar, most preferably 85%, 90% or 95% similar to the amino acid sequence as given in SEQ ID NO: 10 or 35,

(g) a nucleic acid encoding a protein comprising the amino acid sequence as given in any of SEQ ID NOs: 4, 6, 10, 32 or 35,

(h) a nucleic acid which is degenerated to a nucleic acid as given in any of SEQ ID NOs: 29, 3, 5, 9, 26, 27, 33 or 34 or which is degenerated to a nucleic acid as defined in any of (a) to (g) as a result of the genetic code,

(i) a nucleic acid which is diverging from a nucleic acid encoding a protein as given in any of SEQ ID NOs: 4, 6, 10 or 35 or which is diverging from a nucleic acid as defined in any of (a) to (g) due to the differences in codon usage between the organisms,

(j) a nucleic acid encoding a protein as given in SEQ ID NOs: 4, 6, 10 or 35, or a nucleic acid as defined in (a) to (g) which is diverging due to the differences between alleles,

(k) a nucleic acid encoding an immunologically active fragment of a cytokinin oxidase encoded by a nucleic acid as given in any of SEQ ID NOs: 29, 3, 5, 9, 26, 27, 31, 33 or 34, or an immunologically active fragment of a nucleic acid as defined in any of (a) to (j),

(l) a nucleic acid encoding a functional fragment of a cytokinin oxidase encoded by a nucleic acid as given in any of SEQ ID NOs: 29, 3, 5, 9, 26, 27, 31, 33 or 34, or a functional fragment of a nucleic acid as defined in any of (a) to (j), wherein said fragment has the biological activity of a cytokinin oxidase, and

(m) a nucleic acid encoding a protein as defined in SEQ ID NOs: 4, 6, 10 or 35,

provided that said nucleic acid is not the nucleic acid as deposited under any of the following Genbank accession numbers: AC005917, AB024035, and AC023754

The invention also relates to an isolated nucleic acid of the invention which is DNA, cDNA, genomic DNA or synthetic DNA, or RNA wherein T is replaced by U.

The invention also relates to a nucleic acid molecule of at least 15 nucleotides in length hybridizing specifically with or specifically amplifying a nucleic acid of the invention.

Different cytokinin forms may have differing roles to play in the various developmental processes. Thus, differential effects of CKX1, CKX2 , CKX 3 and CKX4 may relate to distinct effects on the pools of different cytokinins. For example, CKX1 and CKX3 mostly promote root elongation and branching, while CKX2 and CKX4 primarily stimulate the formation of adventitious roots . In addition, CKX1 and CKX3 increase seed size and weight to a greater degree than CKX2 and CKX4. Without being bound to a particular mode of action, this differential effect on cytokine pools may result from some differences in substrate specificity or from differential compartmentation of cytokinin oxidases in the cell (predicted to be mitochondrial for CKX1 and CKX3, while extracellular for CKX 2, CKX4, CKX5, and CKX6).

According to another embodiment, the invention also relates to a vector comprising a nucleic acid of the invention. In a preferred embodiment, said vector is an expression vector wherein the nucleic acid is operably linked to one or more control sequences allowing the expression of said sequence in prokaryotic and/or eukaryotic host cells.

It should be understood that for expression of the cytokinin oxidase genes of the invention in monocots, a nucleic acid sequence corresponding to the cDNA

sequence should be used to avoid mis-splicing of introns in monocots. Preferred cDNA sequences to be expressed in monocots have a nucleic acid sequence as represented in any of SEQ ID NOs: 25 to 30 and 34.

The invention also relates to a host cell containing any of the nucleic acid molecules or vectors of the invention. Said host cell is chosen from the group comprising bacterial, insect, fungal, plant or animal cells.

Another embodiment of the invention relates to an isolated polypeptide encodable by a nucleic acid of the invention, or a homologue or a derivative thereof, or an immunologically active or a functional fragment thereof. Preferred polypeptides of the invention comprise the amino acid sequences as represented in any of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 32 and 35, or a homologue or a derivative thereof, or an immunologically active and/or functional fragment thereof. In an even more preferred embodiment, the invention relates to a polypeptide which has an amino acid sequence as given in SEQ ID: NO 2, 4, 6, 8, 10,12 or 35, or a homologue or a derivative thereof, or an immunologically active and/or functional fragment thereof. Preferred functional fragments thereof are those fragments which are devoid of their signal peptide.

According to yet another embodiment, the invention relates to a method for producing a polypeptide of the invention comprising culturing a host cell of the invention under conditions allowing the expression of the polypeptide and recovering the produced polypeptide from the culture.

The invention also relates to an antibody specifically recognizing a polypeptide of the invention or a specific epitope thereof.

The invention further relates to a method for the production of transgenic plants, plant cells or plant tissues comprising the introduction of a nucleic acid molecule of the invention in an expressible format or a vector of the invention in said plant, plant cell or plant tissue.

The invention also relates to a method for the production of altered plants, plant cells or plant tissues comprising the introduction of a polypeptide of the invention directly into a cell, a tissue or an organ of said plant.

According to another embodiment, the invention relates to a method for effecting the expression of a polypeptide of the invention comprising the introduction of a nucleic acid molecule of the invention operably linked to one or more control sequences or a vector of the invention stably into the genome of a plant cell. The invention further relates to the method as described above further comprising regenerating a plant from said plant cell.

The invention also relates to a transgenic plant cell comprising a nucleic acid sequence of the invention which is operably linked to regulatory elements allowing transcription and/or expression of said nucleic acid in plant cells or obtainable by a method as explained above.

According to another preferred embodiment, the invention relates to a transgenic plant cell as described hereinabove wherein the nucleic acid of the invention is stably integrated into the genome of said plant cell.

The invention further relates to a transgenic plant or plant tissue comprising plant cells as herein described and also to a harvestable part of said transgenic plant, preferably selected from the group consisting of seeds, leaves, fruits, stem cultures, roots, tubers, rhizomes and bulbs. The invention also relates to the progeny derived from any of said transgenic plants or plant parts.

According to another embodiment, the invention relates to a method for stimulating root growth comprising expression of a nucleic acid of the invention or comprising expression of another protein that reduces the level of active cytokinins in plants or plant parts.

In another aspect of the invention, there is provided a method of increasing seed size and/or weight. The method comprises increasing the level or activity of a cytokinin oxidase in a plant or increasing the level or activity of a protein that reduces the level of active cytokinins in a plant or plant part, preferably seeds.

Various parts (organs) of the seed may also be increased in size and/or weight such as e.g., embryo, endosperm, seed coat, or aleurone. For example, in accordance with the present invention, there is provided a method of increasing embryo size and/or weight. The method comprises increasing the level or activity of a cytokinin oxidase in a plant or increasing the level or activity of a protein that reduces the level of active cytokinins in a plant or plant part, preferably embryos.

In still another aspect of the invention, there is provided a method of increasing cotyledon size and/or weight. The method comprises increasing the level or activity of a cytokinin oxidase in a plant or increasing the level or activity of a protein that reduces the level of active cytokinins in a plant or plant part, preferably cotyledons.

In accordance with the methods of increasing seed size and/or weight, there is a resultant increase in the speed of growth of seedlings or an increase in early vigor. Increases in yield are also obtained. Similarly, in accordance with the methods of increasing embryo size and/or weight, or cotyledon size and/or weight, there is a resultant increase in speed of growth of seedlings or an increase in early vigor. In many cases, increases in yield are also obtained. Increases in growth of seedlings or early vigor is often associated with increased stress tolerance. For example, faster development of seedlings, including the root systems of seedlings upon germination is critical for survival particularly under adverse conditions such as drought.

Any nucleotide sequence encoding a polypeptide with cytokinin oxidase activity may be used in the methods of the invention. For example, any of the various sequences provided herein encoding a polypeptide with cytokinin oxidase activity may be used in the methods of increasing seed, embryo, or cotyledon size and/or weight.

Preferably, transgenic plants are produced which express a nucleic acid as set forth in any of SEQ ID NOs:1, 5, 25, or 27 or an ortholog of said nucleic acid. Preferably, the ortholog is derived from a related species of the transgenic plant.

Even more preferably, the ortholog is specific (native or endogenous) to the species of the transgenic plant.

As described above, promoters which control expression specifically, or preferentially may be used in the methods of the invention. Thus, where increases in seed size or weight are desired, a seed-specific promoter may be used. Where increases in embryo size or weight are desired, an embryo-specific promoter may be used. Where increases in cotyledon size or weight is desired, a promoter which controls expression in cotyledons is preferred. Such promoters are well known, widely available and listed herein in e.g., Table 4.

In another embodiment, the invention relates to a method for increasing seed size or seed weight, or both, said method comprising expression of a nucleic acid of the invention or comprising expression of another protein that reduces the level of active cytokinins in plants or plant parts

In yet another embodiment, the invention relates to a method for increasing embryo size or weight, or both, said method comprising expression of a nucleic acid of the invention or comprising expression of another protein that reduces the level of active cytokinins in plants or plant parts.

In still another embodiment, the invention relates to a method for increasing cotyledon size comprising expression of a nucleic acid of the invention or comprising expression of another protein that reduces the level of active cytokinins in plants or plant parts. Localized expression of a subject cytokinin oxidase gene or part thereof, or of another protein that reduces the level of active cytokinins in plants or plant parts leads to enhanced growth of cotyledons. In species having cotyledons as storage organs, such enhanced growth of cotyledons leads to enhanced yields and/or to enhanced growth performance of seedlings. Further in this regard, carbohydrates, lipids and proteins are all stored within seeds and are metabolized during germination in order to provide energy and metabolites during early growth of the plant. Seed size is often associated with early vigor, since larger seeds contain more carbohydrates, lipids and proteins and thus confer faster growth. Thus, the methods of the present invention lead to

faster growth of seedlings. Such early vigor is associated with enhanced stress tolerance. For example, faster development of a plant's root system is critical for survival, particularly under adverse conditions, such as drought. Early vigor is also related to enhanced yield and shortened time to flowering.

A plant cell or tissue culture is an artificially produced culture of plants cells or plant tissues that is grown in a special medium, either liquid or solid, which provides these plant cells or tissues with all requirements necessary for growth and/or production of certain compounds. Plant cell and/or tissue cultures can be used for the rapid propagation of plants and for the production of transgenic plant to name a few examples. Root formation can be difficult for some explants or under some conditions in said cultures and expression of a cytokinin oxidase gene in said cultured plant cells or tissue(s) can be used to enhance root formation. Plant cell and/or tissue culture can also be used for the industrial production of valuable compounds. Possible production compounds are pharmaceuticals, pesticides, pigments, cosmetics, perfumes, food additives, etc. An example of such a product is shikonin, which is produced by the roots of the plant *Lithospermum erythrorhizon*. An example of a plant tissue culture is a hairy root culture, which is an artificially produced mass of hairy roots. Roots of *L. erythrorhizon* are difficult to collect in large numbers and by preparing hairy root cultures, the end product shikonin could be industrially prepared at a faster rate than would normally occur. As disclosed herein, expression of cytokinin oxidases enhances root growth and development and can therefore be used advantageously in said plant cell and tissue culture procedures. Therefore, according to another embodiment of this invention, a method is provided for stimulating root growth and development comprising expression of a nucleic acid encoding a plant cytokinin oxidase, preferably a cytokinin oxidase of the invention, in a transgenic plant cell or tissue culture comprising said transgenic plant cells.

The invention further relates to a method for enhancing the formation of lateral or adventitious roots comprising expression of a nucleic acid of the invention or comprising expression of another protein that reduces the level of active cytokinins in plants or plant parts.

The invention also relates to method for altering root geotropism comprising altering the expression of a nucleic acid of the invention or comprising expression of another protein that that reduces the level of active cytokinins in plants or plant parts.

The invention also relates to methods for enhancing early vigor and/or for modifying root/shoot ratio and/or for improving resistance to lodging and/or for increasing drought tolerance and/or for promoting *in vitro* propagation of explants comprising expression of a nucleic acid of the invention comprising expression of another protein that reduces the level of active cytokinins in plants or plant parts.

The invention further relates to methods for increasing the root size or the size of the root meristem comprising expression of a nucleic acid of the invention or comprising expression of another protein that reduces the level of active cytokinins in plants or plant parts, preferably in roots.

According to yet another embodiment, the invention relates to a method for increasing the size of the shoot meristem comprising downregulation of expression of a nucleic acid of the invention, preferably in shoots.

According to a preferred embodiment the invention relates to a method for delaying leaf senescence comprising downregulation of expression of any of the cytokinin oxidases of the invention in leaves, preferably in senescing leaves. Also the invention relates to a method for altering leaf senescence comprising expression of one of the cytokinin oxidases in senescing leaves.

The invention also relates to methods for increasing leaf thickness comprising expression of a nucleic acid of the invention or comprising expression of another protein that reduces the level of active cytokinins in plants or plant parts, preferably in leaves.

The invention also relates to a method for reducing the vessel size comprising expression of a nucleic acid of the invention or comprising expression of another protein that reduces the level of active cytokinins in plants or plant parts, preferably in vessels.

The invention further relates to a method for increasing the vessel size comprising downregulation of expression of a nucleic acid of the invention in plants or plant parts.

According to another embodiment, the invention relates to a method for improving standability of seedlings comprising expression of a nucleic acid of the invention or comprising expression of another protein that reduces the level of active cytokinins in seedlings.

Furthermore, the invention relates to any of the above described methods, said method leading to an increase in yield.

The invention further relates to any of the methods of the invention wherein said expression of said nucleic acid occurs under the control of a strong constitutive promoter. With respect to those aspects of the invention having effects on plant roots such as e.g., methods for stimulating root growth, enhancing the formation of lateral or adventitious roots, or for altering root geotropism, preferably, expression of a subject nucleic acid preferably occurs under the control of a promoter that is preferentially expressed in roots. In Table 5 a non-exhaustive list of root specific promoters is included. A preferred promoter to be used in the methods of the invention is the root clavata homolog promoter, having a sequence as given in SEQ ID NO: 36.

With respect to those aspect of the invention having effects on plant seeds such as e.g., methods for increasing seed size or weight, embryo size or weight, or having effects on plant cotyledons such as methods for increasing cotyledon size of weight, expression of a subject nucleic acid occurs under the control of a promoter that is preferentially expressed in seeds. A seed specific promoter may be one which is expressed in all seed organs or one which shows a preference in expression to one or more organs or tissue such as the embryo, endosperm, or aleurone. Examples of such promoters are set forth herein at Table 4.

According to yet another embodiment, the invention relates to a method for modifying cell fate and/or modifying plant development and/or modifying plant morphology and/or modifying plant biochemistry and/or modifying plant

physiology and/or modifying the cell cycle progression rate comprising the modification of expression in particular cells, tissues or organs of a plant, of a nucleic acid of the invention.

The invention also relates to a method for obtaining enhanced growth, and/or increased yield and/or altered senescence of a plant cell, tissue and/or organ and/or increased frequency of formation of lateral organs in a plant, comprising the ectopic expression of a nucleic acid of the invention.

The invention also relates to a method for promoting and extending cell division activity in cells in adverse growth conditions and/or in stress, comprising the ectopic expression of a nucleic acid sequence of the invention.

According to yet another embodiment, the invention relates to a method for identifying and obtaining proteins interacting with a polypeptide of the invention comprising a screening assay wherein a polypeptide of the invention is used.

In a more preferred embodiment, the invention relates to a method for identifying and obtaining proteins interacting with a polypeptide of the invention comprising a two-hybrid screening assay wherein a polypeptide of the invention as a bait and a cDNA library as prey are used.

The invention further relates to a method for modulating the interaction between a polypeptide of the invention and interacting protein partners obtainable by a method as described above.

In a further embodiment, the invention relates to a method for identifying and obtaining compounds interacting with a polypeptide of the invention comprising the steps of:

(a) providing a two-hybrid system wherein a polypeptide of the invention and an interacting protein partner obtainable by a method as described above,

(b) interacting said compound with the complex formed by the expressed polypeptides as defined in a), and,

(c) performing (real-time) measurement of interaction of said compound with said polypeptide or the complex formed by the expressed polypeptides as defined in a).

The invention further relates to a method for identifying compounds or mixtures of compounds which specifically bind to a polypeptide of the invention, comprising:

(a) combining a polypeptide of the invention with said compound or mixtures of compounds under conditions suitable to allow complex formation, and,

(b) detecting complex formation, wherein the presence of a complex identifies a compound or mixture which specifically binds said polypeptide.

The invention also relates to a method as described above wherein said compound or mixture inhibits the activity of said polypeptide of the invention and can be used for the rational design of chemicals.

According to another embodiment, the invention relates to the use of a compound or mixture identified by means of a method as described above as a plant growth regulator or herbicide.

The invention also relates to a method for production of a plant growth regulator or herbicide composition comprising the steps of the compound screening methods described above and formulating the compounds obtained from said steps in a suitable form for the application in agriculture or plant cell or tissue culture.

The invention also relates to a method for increasing branching comprising expression of a nucleic acid of the invention in plants or plant parts, preferably in stems or axillary buds.

The invention also relates to a method for improving lodging resistance comprising expression of a nucleic acid of the invention in plants or plant parts, preferably in stems or axillary buds.

The invention also relates to a method for the design of or screening for growth-promoting chemicals or herbicides comprising the use of a nucleic acid of the invention or a vector of the invention.

According to another embodiment, the invention relates to the use of a nucleic acid molecule of the invention, a vector of the invention or a polypeptide of the invention for increasing yield.

The invention also relates to the use of a nucleic acid molecule of the invention, a vector of the invention or a polypeptide of the invention for stimulating root growth.

The invention also relates to the use of a nucleic acid molecule of the invention, a vector of the invention or a polypeptide of the invention for enhancing the formation of lateral or adventitious roots.

The invention also relates to the use of a nucleic acid molecule of the invention, a vector of the invention or a polypeptide of the invention for altering root geotropism.

The invention also relates to the use of a nucleic acid molecule of the invention, a vector of the invention or a polypeptide of the invention for increasing at least one of seed size, seed weight, embryo size, embryo weight, cotyledon size, and cotyledon weight.

The invention further relates to the use of a nucleic acid molecule of the invention, a vector of the invention or a polypeptide of the invention for enhancing early vigor and/or for modifying root/shoot ratio and/or for improving resistance to lodging and/or for increasing drought tolerance and/or for promoting *in vitro* propagation of explants.

The invention also relates to the use of a nucleic acid molecule of the invention, a recombinant vector of the invention or a polypeptide of the invention for modifying plant development and/or for modifying plant morphology and/or for modifying plant biochemistry and/or for modifying plant physiology.

According to yet another embodiment, the invention relates to a ddiagnostic composition comprising at least a nucleic acid molecule of the invention, a vector of the invention, a polypeptide of the invention or an antibody of the invention.

Another embodiment of the current invention relates to the use of a transgenic rootstock that has an enhanced root growth and development due to expression of a cytokinin oxidase in grafting procedures with a scion to produce a plant or tree with improved agricultural or horticultural characteristics. The scion may be transgenic or non-transgenic. Specific characteristics envisaged by this embodiment are those conferred by root systems and include improved anchoring of the plant/tree in the soil and/or improved uptake of water resulting for example in improved drought tolerance, and/or improved nutrient uptake from the soil and/or improved transport of organic substances throughout the plant and/or enhanced secretion of substances into the soil such as for example phytosiderophores, and/or improved respiration and/or improved disease resistance and/or enhanced yield. An advantage of using *AtCKX* transformed rootstocks for grafting, in addition to their enhanced root system, is the delayed senescence of leaves on the graft, as disclosed herein (see Figure 12 A). Preferred plants or trees for this particular embodiment include plants or trees that do not grow well on their own roots and are grafted in cultivated settings such as commercially profitable varieties of grapevines, citrus, apricot, almond, plum, peach, apple, pear, cherry, walnut, fig, hazel and loquat.

As mentioned *supra*, auxins and cytokinins act as antagonists in certain biological processes. For example, the cytokinin/auxin ratio regulates the production of roots and shoots with a high concentration of auxin resulting in organized roots and a high concentration of cytokinins resulting in shoot production. As disclosed in this invention, expression of cytokinin oxidases in

tobacco and Arabidopsis results in enhanced root development consistent with enhanced auxin effects. Auxins are also involved in the development of fruit. Treatment of female flower parts with auxin results in the development of parthenocarpic fruit in some plant species. Parthenocarpic fruit development has been genetically engineered in several horticultural crop plants through increased biosynthesis of auxins in the female reproductive organs (WO0105985).

Therefore, according to another embodiment, this invention relates to a method for inducing the parthenocarpic trait in plants, said method consisting of downregulating the expression of one or more cytokinin oxidases or of another protein that reduces the level of active cytokinins in plants or plant parts, preferably in the female reproductive organs such as the placenta, ovules and tissues derived therefrom. The DefH9 promoter region from *Antirrhinum majus* or one of its homologues, which confer high expression specificity in placenta and ovules, can be used for this purpose.

Those skilled in the art will be aware that the invention described herein is subject to variations and modifications other than those specifically described. It is to be understood that the invention described herein includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any or more of said steps or features.

The present invention is applicable to any plant, in particular a monocotyledonous plants and dicotyledonous plants including a fodder or forage legume, ornamental plant, food crop, tree, or shrub selected from the list comprising *Acacia spp., Acer spp., Actinidia spp.,Aesculus spp., Agathis australis, Albizia amara, Alsophila tricolor, Andropogon spp., Arachis spp, Areca catechu, Astelia fragrans, Astragalus cicer, Baikiaea plurijuga, Betula spp., Brassica spp., Bruguiera gymnorrhiza, Burkea africana, Butea frondosa, Cadaba farinosa, Calliandra spp, Camellia sinensis, Canna indica, Capsicum spp., Cassia spp., Centroema pubescens, Chaenomeles spp.,Cinnamomum cassia, Coffea arabica, Colophospermum mopane, Coronillia varia, Cotoneaster serotina, Crataegus*

spp., Cucumis spp., Cupressus spp., Cyathea dealbata, Cydonia oblonga, Cryptomeria japonica, Cymbopogon spp., Cynthea dealbata, Cydonia oblonga, Dalbergia monetaria, Davallia divaricata, Desmodium spp., Dicksonia squarosa, Diheteropogon amplectens, Dioclea spp, Dolichos spp., Dorycnium rectum, Echinochloa pyramidalis, Ehrartia spp., Eleusine coracana, Eragrestis spp., Erythrina spp., Eucalyptus spp., Euclea schimperi, Eulalia villosa, Fagopyrum spp., Feijoa sellowiana, Fragaria spp., Flemingia spp, Freycinetia banksii, Geranium thunbergii, Ginkgo biloba, Glycine javanica, Gliricidia spp, Gossypium hirsutum, Grevillea spp., Guibourtia coleosperma, Hedysarum spp., Hemarthia altissima, Heteropogon contortus, Hordeum vulgare, Hyparrhenia rufa, Hypericum erectum, Hyperthelia dissoluta, Indigo incarnata, Iris spp., Leptarrhena pyrolifolia, Lespediza spp., Lettuca spp., Leucaena leucocephala, Loudetia simplex, Lotonus bainesii, Lotus spp., Macrotyloma axillare, Malus spp., Manihot esculenta, Medicago sativa, Metasequoia glyptostroboides, Musa sapientum, Nicotianum spp., Onobrychis spp., Ornithopus spp., Oryza spp., Peltophorum africanum, Pennisetum spp., Persea gratissima, Petunia spp., Phaseolus spp., Phoenix canariensis, Phormium cookianum, Photinia spp., Picea glauca, Pinus spp., Pisum sativum, Podocarpus totara, Pogonarthria fleckii, Pogonarthria squarrosa, Populus spp., Prosopis cineraria, Pseudotsuga menziesii, Pterolobium stellatum, Pyrus communis, Quercus spp., Rhaphiolepsis umbellata, Rhopalostylis sapida, Rhus natalensis, Ribes grossularia, Ribes spp., Robinia pseudoacacia, Rosa spp., Rubus spp., Salix spp., Schyzachyrium sanguineum, Sciadopitys verticillata, Sequoia sempervirens, Sequoiadendron giganteum, Sorghum bicolor, Spinacia spp., Sporobolus fimbriatus, Stiburus alopecuroides, Stylosanthos humilis, Tadehagi spp, Taxodium distichum, Themeda triandra, Trifolium spp., Triticum spp., Tsuga heterophylla, Vaccinium spp., Vicia spp.Vitis vinifera, Watsonia pyramidata, Zantedeschia aethiopica, Zea mays, amaranth, artichoke, asparagus, broccoli, brussel sprout, cabbage, canola, carrot, cauliflower, celery, collard greens, flax, kale, lentil, oilseed rape, okra, onion, potato, rice, soybean, straw, sugarbeet, sugar cane, sunflower, tomato, squash, and tea, amongst others, or the seeds of any plant specifically named above or a tissue, cell or organ culture of any of the above species.

Throughout this specification, unless the context requires otherwise the word “comprise”, and variations such as “comprises” and “comprising”, will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

As used herein, the term “derived from” shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

The terms "protein(s)", "peptide(s)" or "oligopeptide(s)", when used herein refer to amino acids in a polymeric form of any length. Said terms also include known amino acid modifications such as disulphide bond formation, cysteinylation, oxidation, glutathionylation, methylation, acetylation, farnesylation, biotinylation, stearoylation, formylation, lipoic acid addition, phosphorylation, sulphation, ubiquitination, myristoylation, palmitoylation, geranylgeranylation, cyclization (e.g. pyroglutamic acid formation), oxidation, deamidation, dehydration, glycosylation (e.g. pentoses, hexosamines, N-acetylhexosamines, deoxyhexoses, hexoses, sialic acid etc.) and acylation as well as non-naturally occurring amino acid residues, L-amino acid residues and D-amino acid residues.

"Homologues" of a protein of the invention are those peptides, oligopeptides, polypeptides, proteins and enzymes which contain amino acid substitutions, deletions and/or additions relative to the said protein with respect to which they are a homologue, without altering one or more of its functional properties, in particular without reducing the activity of the resulting. For example, a homologue of said protein will consist of a bioactive amino acid sequence variant of said protein. To produce such homologues, amino acids present in the said protein can be replaced by other amino acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment, antigenicity, propensity to form or break α-helical structures or β-sheet structures, and so on. An overview of physical and chemical properties of amino acids is given in Table 1.

Substitutional variants of a protein of the invention are those in which at least one residue in said protein amino acid sequence has been removed and a different residue inserted in its place. Amino acid substitutions are typically of single residues, but may be clustered depending upon functional constraints placed upon the polypeptide; insertions will usually be of the order of about 1-10 amino acid residues and deletions will range from about 1-20 residues. Preferably, amino acid substitutions will comprise conservative amino acid substitutions, such as those described *supra*.

Table 1. Properties of naturally occurring amino acids.

Charge properties / hydrophobicity	Side group	Amino Acid
Nonpolar hydrophobic	Aliphatic aliphatic, S-containing aromatic imino	ala, ile, leu, val met phe, trp pro
polar uncharged	Aliphatic Amide Aromatic Hydroxyl Sulfhydryl	gly asn, gln tyr ser, thr cys
Positively charged	Basic	arg, his, lys
Negatively charged	Acidic	asp, glu

Insertional amino acid sequence variants of a protein of the invention are those in which one or more amino acid residues are introduced into a predetermined site in said protein. Insertions can comprise amino-terminal and/or carboxy-terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Generally, insertions within the amino acid sequence will be smaller than amino or carboxyl terminal fusions, of the order of about 1 to 10 residues. Examples of amino- or carboxy-terminal fusion proteins or peptides include the binding domain or activation domain of a transcriptional activator as used in a two-hybrid system, phage coat proteins, (histidine)$_6$-tag, glutathione S-transferase, protein A, maltose-binding protein, dihydrofolate reductase, Tag•100 epitope (EETARFQPGYRS), c-myc epitope (EQKLISEEDL), FLAG®-epitope (DYKDDDK), lacZ, CMP (calmodulin-binding peptide), HA epitope (YPYDVPDYA), protein C epitope (EDQVDPRLIDGK) and VSV epitope (YTDIEMNRLGK).

Deletional variants of a protein of the invention are characterized by the removal of one or more amino acids from the amino acid sequence of said protein.

Amino acid variants of a protein of the invention may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis and the like, or by recombinant DNA manipulations. The manipulation of DNA sequences to produce variant proteins which manifest as substitutional, insertional or deletional variants are well known in the art. For example, techniques for making substitution mutations at predetermined sites in DNA having known sequence are well known to those skilled in the art, such as by M13 mutagenesis, T7-Gen in vitro mutagenesis kit (USB, Cleveland, OH), QuickChange Site Directed mutagenesis kit (Stratagene, San Diego, CA), PCR-mediated site-directed mutagenesis or other site-directed mutagenesis protocols.

In the current invention "identity" and/or "similarity" percentages between DNA sequences and/or proteins are calculated using computer programs known in the art such as the DNAstar/MegAlign programs in combination with the Clustal method.

"Derivatives" of a protein of the invention are those peptides, oligopeptides, polypeptides, proteins and enzymes which comprise at least about five contiguous amino acid residues of said polypeptide but which retain the biological activity of said protein. A "derivative" may further comprise additional naturally-occurring, altered glycosylated, acylated or non-naturally occurring amino acid residues compared to the amino acid sequence of a naturally-occurring form of said polypeptide. Alternatively or in addition, a derivative may comprise one or more non-amino acid substituents compared to the amino acid sequence of a naturally-occurring form of said polypeptide, for example a reporter molecule or other ligand, covalently or non-covalently bound to the amino acid sequence such as, for example, a reporter molecule which is bound thereto to facilitate its detection.

With "immunologically active" is meant that a molecule or specific fragments thereof such as specific epitopes or haptens are recognized by, i.e. bind to antibodies. Specific epitopes may be determined using, for example, peptide scanning techniques as described in Geysen *et al.* (1996) (Geysen, H.M., Rodda,

S.J. and Mason, T.J. (1986). A priori delineation of a peptide which mimics a discontinuous antigenic determinant. *Mol. Immunol.* **23**, 709-715.).

The term "fragment of a sequence" or "part of a sequence" means a truncated sequence of the original sequence referred to. The truncated sequence (nucleic acid or protein sequence) can vary widely in length; the minimum size being a sequence of sufficient size to provide a sequence with at least a comparable function and/or activity or the original sequence referred to (e. g. "functional fragment"), while the maximum size is not critical. In some applications, the maximum size usually is not substantially greater than that required to provide the desired activity and/or function(s) of the original sequence. Typically, the truncated amino acid sequence will range from about 5 to about 60 amino acids in length. More typically, however, the sequence will be a maximum of about 50 amino acids in length, preferably a maximum of about 60 amino acids. It is usually desirable to select sequences of at least about 10, 12 or 15 amino acids, up to a maximum of about 20 or 25 amino acids.

Functional fragments can also include those comprising an epitope which is specific for the proteins according to the invention. Preferred functional fragments have a length of at least, for example, 5, 10, 25, 100, 150 or 200 amino acids.

It should thus be understood that functional fragments can also be immunologically active fragments or not.

In the context of the current invention are embodied homologues, derivatives and/or immunologically active and/or functional fragments of the cytokinin oxidases as defined supra. Particularly preferred homologues, derivatives and/or immunologically active and/or functional fragments of the cytokinin oxidase proteins which are contemplated for use in the current invention are derived from plants, more specifically from *Arabidopsis thaliana*, even more specifically said cytokinin oxidases are the *Arabidopsis thaliana* (At)CKX, or are capable of being expressed therein. The present invention clearly contemplates the use of functional homologues or derivatives and/or immunologically active

fragments of the AtCKX proteins and is not to be limited in application to the use of a nucleotide sequence encoding one of said AtCKX proteins.

Any of said proteins, polypeptides, peptides and fragments thereof can be produced in a biological system, e.g. a cell culture. Alternatively any of said proteins, polypeptides, peptides and fragments thereof can be chemically manufactured e.g. by solid phase peptide synthesis. Said proteins or fragments thereof can be part of a fusion protein as is the case in e.g. a two-hybrid assay which enables e.g. the identification of proteins interacting with a cytokinin oxidase according to the invention.

The proteins or fragments thereof are furthermore useful e.g. to modulate the interaction between a cytokinin oxidase according to the invention and interacting protein partners obtained by a method of the invention. Chemically synthesized peptides are particularly useful e.g. as a source of antigens for the production of antisera and/or antibodies.

"Antibodies" include monoclonal, polyclonal, synthetic or heavy chain camel antibodies as well as fragments of antibodies such as Fab, Fv or scFv fragments. Monoclonal antibodies can be prepared by the techniques as described in e.g. Liddle and Cryer (1991) which comprise the fusion of mouse myeloma cells to spleen cells derived from immunized animals. Furthermore, antibodies or fragments thereof to a molecule or fragments thereof can be obtained by using methods as described in e.g. Harlow and Lane (1988). In the case of antibodies directed against small peptides such as fragments of a protein of the invention, said peptides are generally coupled to a carrier protein before immunization of animals. Such protein carriers include keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), ovalbumin and Tetanus toxoid. The carrier protein enhances the immune response of the animal and provides epitopes for T-cell receptor binding sites. The term "antibodies" furthermore includes derivatives thereof such as labeled antibodies. Antibody labels include alkaline phosphatase, PKH2, PKH26, PKH67, fluorescein (FITC), Hoechst 33258, R-phycoerythrin (PE), rhodamine (TRITC), Quantum Red, Texas Red, Cy3, biotin, agarose, peroxidase and gold spheres. Tools in molecular biology relying on antibodies

against a protein include protein gel blot analysis, screening of expression libraries allowing gene identification, protein quantitative methods including ELISA and RIA, immunoaffinity purification of proteins, immunoprecipitation of proteins (see e.g. Example 6) and immunolocalization. Other uses of antibodies and especially of peptide antibodies include the study of proteolytic processing (Loffler et al. 1994, Woulfe et al. 1994), determination of protein active sites (Lerner 1982), the study of precursor and post-translational processing (Baron and Baltimore 1982, Lerner et al. 1981, Semier et al. 1982), identification of protein domains involved in protein-protein interactions (Murakami et al. 1992) and the study of exon usage in gene expression (Tamura et al. 1991).

Embodied in the current invention are antibodies specifically recognizing a cytokinin oxidase or homologue, derivative or fragment thereof as defined supra. Preferably said cytokinin oxidase is a plant cytokinin oxidase, more specifically one of the *Arabidopsis thaliana* cytokinin oxidases (AtCKX).

The terms "gene(s)", "polynucleotide(s)", "nucleic acid(s)", "nucleic acid sequence(s)", "nucleotide sequence(s)", or "nucleic acid molecule(s)", when used herein refer to nucleotides, either ribonucleotides or deoxyribonucleotides or a combination of both, in a polymeric form of any length. Said terms furthermore include double-stranded and single-stranded DNA and RNA. Said terms also include known nucleotide modifications such as methylation, cyclization and 'caps' and substitution of one or more of the naturally occurring nucleotides with an analog such as inosine. Modifications of nucleotides include the addition of acridine, amine, biotin, cascade blue, cholesterol, Cy3®, Cy5®, Cy5.5® Dabcyl, digoxigenin, dinitrophenyl, Edans, 6-FAM, fluorescein, 3'-glyceryl, HEX, IRD-700, IRD-800, JOE, phosphate psoralen, rhodamine, ROX, thiol (SH), spacers, TAMRA, TET, AMCA-S®, SE, BODIPY®, Marina Blue®, Pacific Blue®, Oregon Green®, Rhodamine Green®, Rhodamine Red®, Rhodol Green® and Texas Red®. Polynucleotide backbone modifications include methylphosphonate, 2'-OMe-methylphosphonate RNA, phosphorothiorate, RNA, 2'-OMeRNA. Base modifications include 2-amino-dA, 2-aminopurine, 3'-(ddA), 3'dA(cordycepin), 7-deaza-dA, 8-Br-dA, 8-oxo-dA, N^6-Me-dA, abasic site (dSpacer), biotin dT, 2'-OMe-5Me-C, 2'-OMe-propynyl-C, 3'-(5-Me-dC), 3'-(ddC), 5-Br-dC, 5-I-dC, 5-

Me-dC, 5-F-dC, carboxy-dT, convertible dA, convertible dC, convertible dG, convertible dT, convertible dU, 7-deaza-dG, 8-Br-dG, 8-oxo-dG, O^6-Me-dG, S6-DNP-dG, 4-methyl-indole, 5-nitroindole, 2'-OMe-inosine, 2'-dI, 0^6-phenyl-dI, 4-methyl-indole, 2'-deoxynebularine, 5-nitroindole, 2-aminopurine, dP(purine analogue), dK(pyrimidine analogue), 3-nitropyrrole, 2-thio-dT, 4-thio-dT, biotin-dT, carboxy-dT, O^4-Me-dT, O^4-triazol dT, 2'-OMe-propynyl-U, 5-Br-dU, 2'-dU, 5-F-dU, 5-I-dU, O^4-triazol dU. Said terms also encompass peptide nucleic acids (PNAs), a DNA analogue in which the backbone is a pseudopeptide consisting of N-(2-aminoethyl)-glycine units rather than a sugar. PNAs mimic the behavior of DNA and bind complementary nucleic acid strands. The neutral backbone of PNA results in stronger binding and greater specificity than normally achieved. In addition, the unique chemical, physical and biological properties of PNA have been exploited to produce powerful biomolecular tools, antisense and antigene agents, molecular probes and biosensors.

The present invention also advantageously provides nucleic acid sequences of at least approximately 15 contiguous nucleotides of a nucleic acid according to the invention and preferably from 15 to 50 nucleotides. These sequences may, advantageously be used as probes to specifically hybridize to sequences of the invention as defined above or primers to initiate specific amplification or replication of sequences of the invention as defined above, or the like. Such nucleic acid sequences may be produced according to techniques well known in the art, such as by recombinant or synthetic means. They may also be used in diagnostic kits or the like for detecting the presence of a nucleic acid according to the invention. These tests generally comprise contacting the probe with the sample under hybridising conditions and detecting the presence of any duplex or triplex formation between the probe and any nucleic acid in the sample.

Advantageously, the nucleic acid sequences, according to the invention may be produced using such recombinant or synthetic means, such as for example using PCR cloning mechanisms which generally involve making a pair of primers, which may be from approximately 15 to 50 nucleotides to a region of the gene which is desired to be cloned, bringing the primers into contact with mRNA, cDNA or genomic DNA from a cell, performing a polymerase chain reaction

under conditions which bring about amplification of the desired region, isolating the amplified region or fragment and recovering the amplified DNA. Generally, such techniques as defined herein are well known in the art, such as described in Sambrook et al. (Molecular Cloning: a Laboratory Manual, 1989).

A "coding sequence" or "open reading frame" or "ORF" is defined as a nucleotide sequence that can be transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate control sequences or regulatory sequences, i.e. when said coding sequence or ORF is present in an expressible format. Said coding sequence of ORF is bounded by a 5' translation start codon and a 3' translation stop codon. A coding sequence or ORF can include, but is not limited to RNA, mRNA, cDNA, recombinant nucleotide sequences, synthetically manufactured nucleotide sequences or genomic DNA. Said coding sequence or ORF can be interrupted by intervening nucleic acid sequences.

Genes and coding sequences essentially encoding the same protein but isolated from different sources can consist of substantially divergent nucleic acid sequences. Reciprocally, substantially divergent nucleic acid sequences can be designed to effect expression of essentially the same protein. Said nucleic acid sequences are the result of e.g. the existence of different alleles of a given gene, of the degeneracy of the genetic code or of differences in codon usage. Thus, as indicated in Table 2, amino acids such as methionine and tryptophan are encoded by a single codon whereas other amino acids such as arginine, leucine and serine can each be translated from up to six different codons. Differences in preferred codon usage are illustrated in Table 3 for *Agrobacterium tumefaciens* (a bacterium), *A. thaliana*, *M. sativa* (two dicotyledonous plants) and *Oryza sativa* (a monocotyledonous plant). To extract one example, the codon GGC (for glycine) is the most frequently used codon in *A. tumefaciens* (36.2 ‰), is the second most frequently used codon in *O. sativa* but is used at much lower frequencies in *A. thaliana* and *M. sativa* (9 ‰ and 8.4 ‰, respectively). Of the four possible codons encoding glycine (see Table 2), said GGC codon is most preferably used in *A. tumefaciens* and *O. sativa*. However, in *A. thaliana* this is the GGA (and GGU) codon whereas in *M. sativa* this is the GGU (and GGA) codon.

DNA sequences as defined in the current invention can be interrupted by intervening sequences. With "intervening sequences" is meant any nucleic acid sequence which disrupts a coding sequence comprising said inventive DNA sequence or which disrupts the expressible format of a DNA sequence comprising said inventive DNA sequence. Removal of the intervening sequence restores said coding sequence or said expressible format. Examples of intervening sequences include introns and mobilizable DNA sequences such as transposons. With "mobilizable DNA sequence" is meant any DNA sequence that can be mobilized as the result of a recombination event.

Table 2. Degeneracy of the genetic code.

Amino Acid	Three-letter code	One-letter code	Possible codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Asparagine	Asn	N	AAC	AAU				
Aspartic Acid	Asp	D	GAC	GAU				
Cysteine	Cys	C	UGC	UGU				
Glutamic Acid	Glu	E	GAA	GAG				
Glutamine	Gln	Q	CAA	CAG				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Lysine	Lys	K	AAA	AAG				
Methionine	Met	M	AUG					
Phenylalanine	Phe	F	UUC	UUU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				
Valine	Val	V	GUA	GUC	GUG	GUU		
			Possible "STOP" codons					
			UAA	UAG	UGA			

Table 3. Usage of the indicated codons in the different organisms given as frequency per thousand codons (http://www.kazusa.or.jp/codon).

Codon	*Agrobacterium tumefaciens*	*Arabidopsis thaliana*	*Medicago sativa*	*Oryza sativa*
UUU	13.9	22.5	24.1	11.3
UUC	24.3	20.7	16.9	26.3
UUA	3.5	12.9	10.4	4.7
UUG	13.2	21.0	22.4	11.8
UCU	7.0	24.6	19.8	10.1
UCC	14.8	10.8	7.7	16.9
UCA	7.4	17.8	17.2	9.7
UCG	18.2	8.9	3.2	10.8
UAU	12.3	15.2	16.6	9.2
UAC	10.3	13.7	14.0	20.6
UAA	0.9	0.9	1.2	0.9
UAG	0.6	0.5	0.8	0.8
UGU	3.0	10.8	10.6	5.0
UGC	7.4	7.2	5.8	14.3
UGA	1.8	1.0	0.8	1.3
UGG	12.2	12.7	10.0	12.8
CUU	19.1	24.3	28.3	14.6
CUC	25.7	15.9	12.0	28.0
CUA	5.2	10.0	8.8	5.7
CUG	31.6	9.9	8.5	22.1
CCU	7.7	18.3	23.2	11.8
CCC	10.6	5.3	5.3	12.5
CCA	8.9	16.1	22.6	12.2
CCG	20.7	8.3	3.6	16.7
CAU	10.6	14.0	14.6	9.2
CAC	9.1	8.7	9.1	14.6
CAA	11.2	19.7	23.2	11.9
CAG	24.9	15.2	12.3	24.6
CGU	12.2	8.9	10.1	6.8
CGC	25.5	3.7	4.2	15.9
CGA	8.2	6.2	4.2	4.2
CGG	13.2	4.8	1.8	9.7
AUU	15.4	22.0	29.4	13.8
AUC	36.9	18.5	14.7	25.5
AUA	6.2	12.9	11.7	7.2
AUG	24.7	24.5	21.7	24.4
ACU	6.4	17.8	20.8	10.3
ACC	20.9	10.3	11.7	18.6
ACA	9.1	15.9	18.9	10.0

ACG	18.8	7.6	2.8	10.8
AAU	13.5	22.7	25.0	12.9
AAC	18.7	20.9	18.7	25.1
AAA	13.6	31.0	32.2	12.0
AAG	24.4	32.6	35.1	39.4
AGU	5.7	14.0	12.6	7.3
AGC	15.8	11.1	8.8	16.9
AGA	5.3	18.7	13.6	7.7
AGG	6.5	10.9	11.7	14.9
GUU	16.6	27.3	34.7	15.0
GUC	29.3	12.7	9.9	22.8
GUA	6.1	10.1	10.0	5.7
GUG	19.7	17.5	16.5	25.0
GCU	17.4	28.0	34.6	19.8
GCC	35.8	10.3	11.4	33.2
GCA	19.5	17.6	25.9	15.6
GCG	31.7	8.8	3.4	25.3
GAU	25.8	36.8	40.0	21.5
GAC	28.0	17.3	15.5	31.6
GAA	29.9	34.4	35.9	17.1
GAG	26.3	32.2	27.4	41.1
GGU	16.5	22.2	28.7	16.3
GGC	36.2	9.0	8.4	34.7
GGA	12.5	23.9	27.3	15.0
GGG	11.3	10.2	7.4	16.6

"Hybridization" is the process wherein substantially homologous complementary nucleotide sequences anneal to each other. The hybridization process can occur entirely in solution, i.e. both complementary nucleic acids are in solution. Tools in molecular biology relying on such a process include PCR, subtractive hybridization and DNA sequence determination. The hybridization process can also occur with one of the complementary nucleic acids immobilized to a matrix such as magnetic beads, Sepharose beads or any other resin. Tools in molecular biology relying on such a process include the isolation of poly (A+) mRNA. The hybridization process can furthermore occur with one of the complementary nucleic acids immobilized to a solid support such as a nitrocellulose or nylon membrane or immobilized by e.g. photolithography to e.g. a silicious glass support (the latter known as nucleic acid arrays or microarrays or as nucleic acid chips). Tools in molecular biology relying on such a process include RNA and DNA gel blot analysis, colony hybridization, plaque

hybridization and microarray hybridization. In order to allow hybridization to occur, the nucleic acid molecules are generally thermally or chemically (e.g. by NaOH) denatured to melt a double strand into two single strands and/or to remove hairpins or other secondary structures from single stranded nucleic acids. The stringency of hybridization is influenced by conditions such as temperature, salt concentration and hybridization buffer composition. High stringency conditions for hybridization include high temperature and/or low salt concentration (salts include NaCl and Na3-citrate) and/or the inclusion of formamide in the hybridization buffer and/or lowering the concentration of compounds such as SDS (detergent) in the hybridization buffer and/or exclusion of compounds such as dextran sulfate or polyethylene glycol (promoting molecular crowding) from the hybridization buffer. Conventional hybridization conditions are described in e.g. Sambrook et al. (1989) but the skilled craftsman will appreciate that numerous different hybridization conditions can be designed in function of the known or the expected homology and/or length of the nucleic acid sequence. Sufficiently low stringency hybridization conditions are particularly preferred to isolate nucleic acids heterologous to the DNA sequences of the invention defined supra. Elements contributing to said heterology include allelism, degeneration of the genetic code and differences in preferred codon usage as discussed supra.

The term "specifically hybridizing" or "hybridizing specifically" refers to the binding, duplexing, or hybridizing of a molecule to a particular nucleotide sequence under medium to stringent conditions when that sequence is presented in a complex mixture e.g., total cellular DNA or RNA.

"Stringent hybridization conditions" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and Northern hybridizations are sequence dependent and are different under different environmental parameters. For example, longer sequences hybridize specifically at higher temperatures. The T_m is the temperature under defined ionic strength and pH, at which 50% of the target sequence hybridizes to a perfectly matched probe. Specificity is typically the function of post-hybridization washes. Critical factors of such washes include the ionic strength and temperature of the final wash solution.

Generally, stringent conditions are selected to be about 50°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. The T_m is dependent upon the solution conditions and the base composition of the probe, and may be calculated using the following equation:

$$
\begin{aligned}
T_m = 79.8^\circ C \quad & + \quad (18.5 \times \mathrm{Log}[Na+]) \\
& + \quad (58.4^\circ C \times \%[G+C]) \\
& - \quad (820 / \#\ \mathrm{bp\ in\ duplex}) \\
& - \quad (0.5 \times \%\ \mathrm{formamide})
\end{aligned}
$$

More preferred stringent conditions are when the temperature is 20°C below T_m, and the most preferred stringent conditions are when the temperature is 10°C below T_m. Nonspecific binding may also be controlled using any one of a number of known techniques such as, for example, blocking the membrane with protein-containing solutions, addition of heterologous RNA, DNA, and SDS to the hybridization buffer, and treatment with RNase.

Wash conditions are typically performed at or below stringency. Generally, suitable stringent conditions for nucleic acid hybridization assays or gene amplification detection procedures are as set forth above. More or less stringent conditions may also be selected.

For the purposes of defining the level of stringency, reference can conveniently be made to Sambrook, J., E.F. Fritsch, et al. 1989 "Molecular Cloning: a Laboratory Manual, 2nd Edition, Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, at 11.45. An example of low stringency conditions is 4-6X SSC/0.1-0.5% w/v SDS at 37°-45° C for 2-3 hours. Depending on the source and concentration of the nucleic acid involved in the hybridization, alternative conditions of stringency may be employed such as medium stringent conditions. Examples of medium stringent conditions include 1-4X SSC/0.25% w/v SDS at ≥ 45° C for 2-3 hours. An example of high stringency conditions includes 0.1-1X SSC/0.1% w/v SDS at 60 C for 1-3 hours. The skilled artisan is aware of various parameters which may be altered during hybridization and

washing and which will either maintain or change the stringency conditions. For example, another stringent hibridization condition is hybridization at 4X SSC at 65° C, followed by a washing in 0.1X SSC at 65° C for about one hour. Alternatively, an exemplary stringent hybridization condition is in 50% formamide, 4XSSC, at 42° C. Still another example of stringent conditions include hybridization at 62° C in 6X SSC, .05X BLOTTO, and washing at 2X SSC, 0.1% SDS at 62° C.

Clearly, the current invention embodies the use of the inventive DNA sequences encoding a cytokinin oxidase, homologue, derivative or immunologically active and/or functional fragment thereof as defined higher in any method of hybridization. The current invention furthermore also relates to DNA sequences hybridizing to said inventive DNA sequences. Preferably said cytokinin oxidase is a plant cytokinin oxidase, more specifically the *Arabidopsis thaliana* (At)CKX.

To effect expression of a protein in a cell, tissue or organ, preferably of plant origin, either the protein may be introduced directly to said cell, such as by microinjection or ballistic means or alternatively, an isolated nucleic acid molecule encoding said protein may be introduced into said cell, tissue or organ in an expressible format.

Preferably, the DNA sequence of the invention comprises a coding sequence or open reading frame (ORF) encoding a cytokinin oxidase protein or a homologue or derivative thereof or an immunologically active and/or functional fragment thereof as defined *supra*. The preferred protein of the invention comprises the amino acid sequence of said cytokinin oxidase. Preferably said cytokinin oxidase is a plant cytokinin oxidase and more specifically a *Arabidopsis thaliana* (At)CKX.

With "vector" or "vector sequence" is meant a DNA sequence which can be introduced in an organism by transformation and can be stably maintained in said organism. Vector maintenance is possible in e.g. cultures of *Escherichia coli*, *A. tumefaciens*, *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*. Other

vectors such as phagemids and cosmid vectors can be maintained and multiplied in bacteria and/or viruses. Vector sequences generally comprise a set of unique sites recognized by restriction enzymes, the multiple cloning site (MCS), wherein one or more non-vector sequence(s) can be inserted.

With "non-vector sequence" is accordingly meant a DNA sequence which is integrated in one or more of the sites of the MCS comprised within a vector.

"Expression vectors" form a subset of vectors which, by virtue of comprising the appropriate regulatory or control sequences enable the creation of an expressible format for the inserted non-vector sequence(s), thus allowing expression of the protein encoded by said non-vector sequence(s). Expression vectors are known in the art enabling protein expression in organisms including bacteria (e.g. *E. coli*), fungi (e.g. *S. cerevisiae*, *S. pombe*, *Pichia pastoris*), insect cells (e.g. baculoviral expression vectors), animal cells (e.g. COS or CHO cells) and plant cells (e.g. potato virus X-based expression vectors).

The current invention clearly includes any cytokinin oxidase, homologue, derivative and/or immunologically active and/or functional fragment thereof as defined supra. Preferably said cytokinin oxidase is a plant cytokinin oxidase, more specifically a *Arabidopsis thaliana* (At)CKX.

As an alternative to expression vector-mediated protein production in biological systems, chemical protein synthesis can be applied. Synthetic peptides can be manufactured in solution phase or in solid phase. Solid phase peptide synthesis (Merrifield 1963) is, however, the most common way and involves the sequential addition of amino acids to create a linear peptide chain. Solid phase peptide synthesis includes cycles consisting of three steps: (i) immobilization of the carboxy-terminal amino acid of the growing peptide chain to a solid support or resin; (ii) chain assembly, a process consisting of activation, coupling and deprotection of the amino acid to be added to the growing peptide chain; and (iii) cleavage involving removal of the completed peptide chain from the resin and removal of the protecting groups from the amino acid side chains. Common approaches in solid phase peptide synthesis include Fmoc/tBu (9-

fluorenylmethyloxycarbonyl/t-butyl) and Boc (t-butyloxycarbonyl) as the amino-terminal protecting groups of amino acids. Amino acid side chain protecting groups include methyl (Me), formyl (CHO), ethyl (Et), acetyl (Ac), t-butyl (t-Bu), anisyl, benzyl (Bzl), trifluroacetyl (Tfa), N-hydroxysuccinimide (ONSu, OSu), benzoyl (Bz), 4-methylbenzyl (Meb), thioanizyl, thiocresyl, benzyloxymethyl (Bom), 4-nitrophenyl (ONp), benzyloxycarbonyl (Z), 2-nitrobenzoyl (NBz), 2-nitrophenylsulphenyl (Nps), 4-toluenesulphonyl (Tosyl,Tos), pentafluorophenyl (Pfp), diphenylmethyl (Dpm), 2-chlorobenzyloxycarbonyl (Cl-Z), 2,4,5-trichlorophenyl, 2-bromobenzyloxycarbonyl (Br-Z), tripheylmethyl (Trityl, Trt), and 2,5,7,8-pentamethyl-chroman-6-sulphonyl (Pmc). During chain assembly, Fmoc or Boc are removed resulting in an activated amino-terminus of the amino acid residue bound to the growing chain. The carboxy-terminus of the incoming amino acid is activated by conversion into a highly reactive ester, e.g. by HBTU. With current technologies (e.g. PerSeptive Biosystems 9050 synthesizer, Applied Biosystems Model 431A Peptide Synthesizer), linear peptides of up to 50 residues can be manufactured. A number of guidelines is available to produce peptides that are suitable for use in biological systems including (i) limiting the use of difficult amino acids such as cys, met, trp (easily oxidized and/or degraded during peptide synthesis) or arg; (ii) minimize hydrophobic amino acids (can impair peptide solubility); and (iii) prevent an amino-terminal glutamic acid (can cyclize to pyroglutamate).

By "expressible format" is meant that the isolated nucleic acid molecule is in a form suitable for being transcribed into mRNA and/or translated to produce a protein, either constitutively or following induction by an intracellular or extracellular signal, such as an environmental stimulus or stress (mitogens, anoxia, hypoxia, temperature, salt, light, dehydration, etc) or a chemical compound such as IPTG (isopropyl-β-D-thiogalactopyranoside) or such as an antibiotic (tetracycline, ampicillin, rifampicin, kanamycin), hormone (e.g. gibberellin, auxin, cytokinin, glucocorticoid, brassinosteroid, ethylene, abscisic acid etc), hormone analogue (indoleacetic acid (IAA), 2,4-D, etc) , metal (zinc, copper, iron, etc), or dexamethasone, amongst others. As will be known to those skilled in the art, expression of a functional protein may also require one or more

post-translational modifications, such as glycosylation, phosphorylation, dephosphorylation, or one or more protein-protein interactions, amongst others. All such processes are included within the scope of the term "expressible format".

Preferably, expression of a protein in a specific cell, tissue, or organ, preferably of plant origin, is effected by introducing and expressing an isolated nucleic acid molecule encoding said protein, such as a cDNA molecule, genomic gene, synthetic oligonucleotide molecule, mRNA molecule or open reading frame, to said cell, tissue or organ, wherein said nucleic acid molecule is placed operably in connection with suitable regulatory or control sequences including a promoter, preferably a plant-expressible promoter, and a terminator sequence.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences derived from a classical eukaryotic genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory or control elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner.

The term "promoter" also includes the transcriptional regulatory sequences of a classical prokaryotic gene, in which case it may include a -35 box sequence and/or a -10 box transcriptional regulatory sequences.

The term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of a nucleic acid molecule in a cell, tissue or organ.

Promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression and/or to alter the spatial expression and/or temporal expression of a nucleic acid molecule to which it is operably connected. Such regulatory elements may be placed adjacent to a heterologous promoter sequence to drive expression of a nucleic acid molecule in response to e.g. copper, glucocorticoids, dexamethasone, tetracycline, gibberellin, cAMP, abscisic acid, auxin, wounding, ethylene, jasmonate or salicylic acid or to

confer expression of a nucleic acid molecule to specific cells, tissues or organs such as meristems, leaves, roots, embryo, flowers, seeds or fruits.

In the context of the present invention, the promoter preferably is a plant-expressible promoter sequence. Promoters that also function or solely function in non-plant cells such as bacteria, yeast cells, insect cells and animal cells are not excluded from the invention. By "plant-expressible" is meant that the promoter sequence, including any additional regulatory elements added thereto or contained therein, is at least capable of inducing, conferring, activating or enhancing expression in a plant cell, tissue or organ, preferably a monocotyledonous or dicotyledonous plant cell, tissue, or organ.

The terms "plant-operable" and "operable in a plant" when used herein, in respect of a promoter sequence, shall be taken to be equivalent to a plant-expressible promoter sequence.

Regulatable promoters as part of a binary viral plant expression system are also known to the skilled artisan (Yadav 1999 – WO9922003; Yadav 2000 – WO0017365).

In the present context, a "regulatable promoter sequence" is a promoter that is capable of conferring expression on a structural gene in a particular cell, tissue, or organ or group of cells, tissues or organs of a plant, optionally under specific conditions, however does generally not confer expression throughout the plant under all conditions. Accordingly, a regulatable promoter sequence may be a promoter sequence that confers expression on a gene to which it is operably connected in a particular location within the plant or alternatively, throughout the plant under a specific set of conditions, such as following induction of gene expression by a chemical compound or other elicitor.

Preferably, the regulatable promoter used in the performance of the present invention confers expression in a specific location within the plant, either constitutively or following induction, however not in the whole plant under any circumstances. Included within the scope of such promoters are cell-specific promoter sequences, tissue-specific promoter sequences, organ-specific promoter

sequences, cell cycle specific gene promoter sequences, inducible promoter sequences and constitutive promoter sequences that have been modified to confer expression in a particular part of the plant at any one time, such as by integration of said constitutive promoter within a transposable genetic element (*Ac, Ds, Spm, En*, or other transposon).

Similarly, the term "tissue-specific" shall be taken to indicate that expression is predominantly in a particular tissue or tissue-type, preferably of plant origin, albeit not necessarily exclusively in said tissue or tissue-type.

Similarly, the term "organ-specific" shall be taken to indicate that expression is predominantly in a particular organ, preferably of plant origin, albeit not necessarily exclusively in said organ.

Similarly, the term "cell cycle specific" shall be taken to indicate that expression is predominantly cyclic and occurring in one or more, not necessarily consecutive phases of the cell cycle albeit not necessarily exclusively in cycling cells, preferably of plant origin.

Those skilled in the art will be aware that an "inducible promoter" is a promoter the transcriptional activity of which is increased or induced in response to a developmental, chemical, environmental, or physical stimulus. Similarly, the skilled craftsman will understand that a "constitutive promoter" is a promoter that is transcriptionally active throughout most, but not necessarily all parts of an organism, preferably a plant, during most, but not necessarily all phases of its growth and development.

Those skilled in the art will readily be capable of selecting appropriate promoter sequences for use in regulating appropriate expression of the cytokinin oxidase protein from publicly-available or readily-available sources, without undue experimentation.

Placing a nucleic acid molecule under the regulatory control of a promoter sequence, or in operable connection with a promoter sequence, means positioning said nucleic acid molecule such that expression is controlled by the promoter

sequence. A promoter is usually, but not necessarily, positioned upstream, or at the 5'-end, and within 2 kb of the start site of transcription, of the nucleic acid molecule which it regulates. In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting (i.e., the gene from which the promoter is derived). As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting (i.e., the gene from which it is derived). Again, as is known in the art, some variation in this distance can also occur.

Examples of promoters suitable for use in gene constructs of the present invention include those listed in Table 4, amongst others. The promoters listed in Table 4 are provided for the purposes of exemplification only and the present invention is not to be limited by the list provided therein. Those skilled in the art will readily be in a position to provide additional promoters that are useful in performing the present invention.

In the case of constitutive promoters or promoters that induce expression throughout the entire plant, it is preferred that such sequences are modified by the addition of nucleotide sequences derived from one or more of the tissue-specific promoters listed in Table 4, or alternatively, nucleotide sequences derived from one or more of the above-mentioned tissue-specific inducible promoters, to confer tissue-specificity thereon. For example, the CaMV 35S promoter may be modified by the addition of maize *Adh1* promoter sequence, to confer anaerobically-regulated root-specific expression thereon, as described previously (Ellis *et al.*, 1987). Another example describes conferring root specific or root abundant gene expression by fusing the CaMV35S promoter to elements of the maize glycine-rich protein GRP3 gene (Feix and Wulff 2000 - WO0015662). Such modifications can be achieved by routine experimentation by those skilled in the art.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in cells derived from viruses, yeasts, molds, bacteria, insects, birds, mammals and plants are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants.

Table 4. Exemplary plant-expressible promoters for use in the performance of the present invention

I: CELL-SPECIFIC,TISSUE-SPECIFIC, AND ORGAN-SPECIFIC PROMOTERS		
GENE SOURCE	**EXPRESSION PATTERN**	**REFERENCE**
α-amylase (*Amy32b*)	aleurone	Lanahan, M.B., *e t al., Plant Cell 4:*203-211, 1992*;* Skriver, K., *et al. Proc. Natl. Acad. Sci. (USA) 88:* 7266-7270, 1991
cathepsin β-like gene	aleurone	Cejudo, F.J., *et al. Plant Molecular Biology 20:*849-856, 1992.
Agrobacterium rhizogenes rolB	cambium	Nilsson *et al., Physiol. Plant. 100*:456-462, 1997
AtPRP4	flowers	http://salus.medium.edu/mmg/tierney/html
chalcone synthase (chsA)	flowers	Van der Meer, *et al., Plant Mol. Biol. 15*, 95-109, 1990.
LAT52	anther	Twell et al Mol. Gen Genet. 217:240-245 (1989)
apetala-3	flowers	
Chitinase	fruit (berries, grapes, etc)	Thomas *et al.* CSIRO Plant Industry, Urrbrae, South Australia, Australia; http://winetitles.com.au/gwrdc/csh95-1.html
rbcs-3A	green tissue (eg leaf)	Lam, E. *et al., The Plant Cell 2:* 857-866, 1990.; Tucker *et al., Plant Physiol. 113:* 1303-1308, 1992.
leaf-specific genes	leaf	Baszczynski, *et al., Nucl. Acid Res. 16:* 4732, 1988.
AtPRP4	leaf	http://salus.medium.edu/mmg/tierney/html
chlorella virus adenine methyltransferase gene promoter	leaf	Mitra and Higgins, 1994, Plant Molecular Biology 26: 85-93
aldP gene promoter from rice	leaf	Kagaya et al., 1995, Molecular and General Genetics 248: 668-674
rbcs promoter from rice or tomato	leaf	Kyozuka et al., 1993, Plant Physiology 102: 991-1000
Pinus cab-6	leaf	Yamamoto *et al., Plant Cell Physiol. 35*:773-778, 1994.

rubisco promoter	leaf	
cab (chlorophyll a/b/binding protein	leaf	
SAM22	senescent leaf	Crowell, *et al., Plant Mol. Biol. 18:* 459-466, 1992.
ltp gene (lipid transfer gene)		*Fleming, et al, Plant J. 2, 855-862.*
R. japonicum nif gene	Nodule	United States Patent No. 4, 803, 165
B. japonicum nifH gene	Nodule	United States Patent No. 5, 008, 194
GmENOD40	Nodule	Yang, *et al., The Plant J. 3:* 573-585.
PEP carboxylase (PEPC)	Nodule	Pathirana, *et al., Plant Mol. Biol. 20:* 437-450, 1992.
Leghaemoglobin (Lb)	Nodule	Gordon, *et al., J. Exp. Bot. 44:* 1453-1465, 1993.
Tungro bacilliform virus gene	phloem	Bhattacharyya-Pakrasi, *et al, The Plant J. 4:* 71-79, 1992.
pollen-specific genes	pollen; microspore	Albani, *et al., Plant Mol. Biol. 15:* 605, 1990; Albani, *et al., Plant Mol. Biol. 16:* 501, 1991)
Zm13	pollen	Guerrero et al Mol. Gen. Genet. 224:161-168 (1993)
apg gene	microspore	Twell et al Sex. Plant Reprod. 6:217-224 (1993)
maize pollen-specific gene	pollen	Hamilton, et al., Plant Mol. Biol. 18: 211-218, 1992.
sunflower pollen-expressed gene	pollen	Baltz, et al., The Plant J. 2: 713-721, 1992.
B. napus pollen-specific gene	pollen;anther; tapetum	Arnoldo, et al., J. Cell. Biochem., Abstract No. Y101, 204, 1992.
root-expressible genes	roots	Tingey, et al., EMBO J. 6: 1, 1987.
tobacco auxin-inducible gene	root tip	Van der Zaal, et al., Plant Mol. Biol. 16, 983, 1991.
β-tubulin	root	Oppenheimer, *et al., Gene 63:* 87, 1988.
tobacco root-specific genes	root	Conkling, et al., Plant Physiol. 93: 1203, 1990.
B. napus G1-3b gene	root	United States Patent No. 5, 401, 836
SbPRP1	roots	Suzuki et al., Plant Mol. Biol. 21: 109-119, 1993.
AtPRP1; AtPRP3	roots; root hairs	http://salus.medium.edu/mmg/tierney/html
RD2 gene	root cortex	http://www2.cnsu.edu/ncsu/research
TobRB7 gene	root vasculature	http://www2.cnsu.edu/ncsu/research

AtPRP4	leaves; flowers; lateral root primordia	http://salus.medium.edu/mmg/tierney/html
seed-specific genes	seed	Simon, *et al., Plant Mol. Biol. 5:* 191, 1985; Scofield, *et al., J. Biol. Chem. 262:* 12202, 1987.; Baszczynski, *et al., Plant Mol. Biol. 14:* 633, 1990.
Brazil Nut albumin	seed	Pearson, et al., Plant Mol. Biol. 18: 235-245, 1992.
Legumin	seed	Ellis, et al., Plant Mol. Biol. 10: 203-214, 1988.
glutelin (rice)	seed	Takaiwa, et al., Mol. Gen. Genet. 208: 15-22, 1986; Takaiwa, et al., FEBS Letts. 221: 43-47, 1987.
Zein	seed	Matzke et al Plant Mol Biol, 14(3):323-32 1990
NapA	seed	Stalberg, *et al, Planta 199:* 515-519, 1996.
wheat LMW and HMW glutenin-1	endosperm	Mol Gen Genet 216:81-90, 1989; NAR 17:461-2, 1989
wheat SPA	seed	Albani *et al,* Plant Cell, 9: 171-184, 1997
wheat α, β, γ-gliadins	endosperm	EMBO 3:1409-15, 1984
barley *Itr1* promoter	endosperm	
barley B1, C, D, hordein	endosperm	Theor Appl Gen 98:1253-62, 1999; Plant J 4:343-55, 1993; Mol Gen Genet 250:750-60, 1996
barley DOF	endosperm	Mena *et al,* The Plant Journal, 116(1): 53-62, 1998
blz2	endosperm	EP99106056.7
synthetic promoter	endosperm	Vicente-Carbajosa *et al., Plant J. 13:* 629-640, 1998.
rice prolamin NRP33	endosperm	Wu *et al,* Plant Cell Physiology 39(8) 885-889, 1998
rice α-globulin Glb-1	endosperm	Wu *et al,* Plant Cell Physiology 39(8) 885-889, 1998
rice OSH1	embryo	Sato *et al,* Proc. Natl. Acad. Sci. USA, 93: 8117-8122, 1996
rice α-globulin REB/OHP-1	endosperm	Nakase *et al.* Plant Mol. Biol. 33: 513-522, 1997
rice ADP-glucose PP	endosperm	Trans Res 6:157-68, 1997
maize ESR gene family	endosperm	Plant J 12:235-46, 1997
sorgum γ-kafirin	endosperm	PMB 32:1029-35, 1996
KNOX	embryo	Postma-Haarsma *et al,* Plant Mol. Biol. 39:257-71, 1999

rice oleosin	embryo and aleuron	Wu *et at,* J. Biochem., 123:386, 1998
sunflower oleosin	seed (embryo and dry seed)	Cummins, *et al.*, Plant Mol. Biol. 19: 873-876, 1992
LEAFY	shoot meristem	Weigel *et al., Cell 69*:843-859, 1992.
Arabidopsis thaliana knat1	shoot meristem	Accession number AJ131822
Malus domestica kn1	shoot meristem	Accession number Z71981
CLAVATA1	shoot meristem	Accession number AF049870
stigma-specific genes	stigma	Nasrallah, et al., Proc. Natl. Acad. Sci. USA 85: 5551, 1988; Trick, *et al., Plant Mol. Biol. 15:* 203, 1990.
class I patatin gene	tuber	Liu et al., Plant Mol. Biol. 153:386-395, 1991.
PCNA rice	meristem	Kosugi *et al, Nucleic Acids Research 19:*1571-1576, 1991; Kosugi S. and Ohashi Y, *Plant Cell 9*:1607-1619, 1997.
Pea TubA1 tubulin	Dividing cells	Stotz and Long, *Plant Mol.Biol.* 41, 601-614. 1999
Arabidopsis cdc2a	cycling cells	Chung and Parish, FEBS Lett, 3;362(2):215-9, 1995
Arabidopsis Rop1A	Anthers; mature pollen + pollen tubes	Li et al. 1998 *Plant Physiol* 118, 407-417.
Arabidopsis AtDMC1	Meiosis-associated	Klimyuk and Jones 1997 *Plant J.* 11, 1-14.
Pea PS-IAA4/5 and PS-IAA6	Auxin-inducible	Wong et al. 1996 *Plant J.* 9, 587-599.
Pea farnesyltransferase	Meristematic tissues; phloem near growing tissues; light- and sugar-repressed	Zhou et al. 1997 *Plant J.* 12, 921-930
Tobacco (*N. sylvestris*) cyclin B1;1	Dividing cells / meristematic tissue	Trehin et al. 1997 *Plant Mol.Biol.* 35, 667-672.
Mitotic cyclins CYS (A-type) and CYM (B-type)	Dividing cells / meristematic tissue	Ito et al. 1997 *Plant J.* 11, 983-992
Arabidopsis cyc1At (=cyc B1;1) and cyc3aAt (A-type)	Dividing cells / meristematic tissue	Shaul et al. 1996 *Proc.Natl.Acad.Sci.U.S.A* 93, 4868-4872.
Arabidopsis tef1 promoter box	Dividing cells / meristematic tissue	Regad et al. 1995 *Mol.Gen.Genet.* 248, 703-711.
Catharanthus roseus cyc07	Dividing cells / meristematic tissue	Ito et al. 1994 *Plant Mol.Biol.* 24, 863-878.

Table 4 (continued). Exemplary plant-expressible promoters for use in the performance of the present invention

II: EXEMPLARY CONSTITUTIVE PROMOTERS		
GENE SOURCE	**EXPRESSION PATTERN**	**REFERENCE**
Actin	constitutive	McElroy *et al,* Plant Cell, 2: 163-171, 1990
CAMV 35S	constitutive	Odell *et al,* Nature, 313: 810-812, 1985
CaMV 19S	constitutive	Nilsson *et al.,* Physiol. Plant. 100:456-462, 1997
GOS2	constitutive	de Pater *et al,* Plant J. 2:837-844, 1992
Ubiquitin	constitutive	Christensen *et al,* Plant Mol. Biol. 18: 675-689, 1992
rice cyclophilin	constitutive	Buchholz *et al,* Plant Mol Biol. 25: 837-843, 1994
maize histone H3	constitutive	Lepetit *et al,* Mol. Gen. Genet. 231:276-285, 1992
alfalfa histone H3	constitutive	Wu *et al.*, Nucleic Acids Res. 17: 3057-3063, 1989; Wu *et al.*, Plant Mol. Biol. 11:641-649, 1988
actin 2	constitutive	An *et al,* Plant J. 10(1); 107-121, 1996

Table 4 (continued). Exemplary plant-expressible promoters for use in the performance of the present invention

III: EXEMPLARY STRESS-INDUCIBLE PROMOTERS		
NAME	**STRESS**	**REFERENCE**
P5CS (delta(1)-pyrroline-5-carboxylate syntase)	salt, water	Zhang et al. Plant Science. 129: 81-89, 1997
cor15a	cold	Hajela et al., Plant Physiol. 93: 1246-1252, 1990
cor15b	cold	Wlihelm et al., Plant Mol Biol. 23:1073-1077, 1993
cor15a (-305 to +78 nt)	cold, drought	Baker et al., Plant Mol Biol. 24: 701-713, 1994
rd29	salt, drought, cold	Kasuga et al., Nature Biotechnology 18:287-291, 1999
heat shock proteins, including artificial promoters containing the heat shock element (HSE)	heat	Barros et al., Plant Mol Biol 19: 665-75, 1992. Marrs et al., Dev Genet.14: 27-41, 1993. Schoffl et al., Mol Gen Gent, 217: 246-53, 1989.
smHSP (small heat shock proteins)	heat	Waters et al, J Experimental Botany 47:325-338, 1996
wcs120	cold	Ouellet et al., FEBS Lett. 423: 324-328, 1998
ci7	cold	Kirch et al., Plant Mol Biol 33: 897-909, 1997
Adh	cold, drought, hypoxia	Dolferus et al., Plant Physiol 105: 1075-87, 1994
pwsi18	water: salt and drought	Joshee et al., Plant Cell Physiol 39: 64-72, 1998
ci21A	cold	Schneider et al., Plant Physiol 113: 335-45, 1997
Trg-31	drought	Chaudhary et al., Plant Mol Biol 30: 1247-57, 1996
Osmotin	osmotic	Raghothama et al., Plant Mol Biol 23: 1117-28, 1993
Rab17	osmotic, ABA	Vilardell et al., Plant Mol Biol 17: 985-93, 1991
LapA	wounding, enviromental	WO99/03977 University of California/INRA

Table 4 (continued). Exemplary plant-expressible promoters for use in the performance of the present invention

IV: EXEMPLARY PATHOGEN-INDUCIBLE PROMOTERS		
NAME	**PATHOGEN**	**REFERENCE**
RB7	Root-knot nematodes (Meloidogyne spp.)	US5760386 - North Carolina State University; Opperman et al (1994) *Science* 263: 221-23.
PR-1, 2, 3, 4, 5, 8, 11	fungal, viral, bacterial	Ward et al (1991) Plant Cell 3: 1085-1094; Reiss et al 1996; Lebel et al (1998), Plant J, 16(2):223-33; Melchers et al (1994), Plant J, 5(4):469-80; Lawton et al (1992), Plant Mol Biol, 19(5):735-43.
HMG2	nematodes	WO9503690 - Virginia Tech Intellectual Properties Inc .
Abi3	Cyst nematodes (Heterodera spp.)	Unpublished
ARM1	nematodes	Barthels et al., (1997) The Plant Cell 9, 2119-2134. WO 98/31822 – Plant Genetic Systems
Att0728	nematodes	Barthels et al., (1997) The Plant Cell 9, 2119-2134. PCT/EP98/07761
Att1712	nematodes	Barthels et al., (1997) The Plant Cell 9, 2119-2134. PCT/EP98/07761
Gst1	Different types of pathogens	Strittmatter et al (1996) Mol. Plant-Microbe Interact. 9, 68-73.
LEMMI	nematodes	*WO 92/21757 – Plant Genetic Systems*
CLE	geminivirus	*PCT/EP99/03445 - CINESTAV*
PDF1.2	Fungal including *Alternaria brassicicola* and *Botrytis cinerea*	Manners et al (1998), Plant Mol Biol, 38(6):1071-80.
Thi2.1	Fungal – *Fusarium oxysporum f sp. matthiolae*	Vignutelli et al (1998) Plant J;14(3):285-95
DB#226	nematodes	Bird and Wilson (1994) Mol. Plant-Microbe Interact., 7, 419-42 WO 95.322888
DB#280	nematodes	Bird and Wilson (1994) Mol. Plant-

		Microbe Interact., 7, 419-42 WO 95.322888
Cat2	nematodes	Niebel et al (1995) Mol Plant Microbe Interact 1995 May-Jun;8(3):371-8
□Tub	nematodes	Aristizabal et al (1996), 8th International Congress on Plant-Microbe Interaction, Knoxville US B-29
SHSP	nematodes	Fenoll et al (1997) In: Cellular and molecular aspects of plant-nematode interactions. Kluwer Academic, C. Fenoll, F.M.W. Grundler and S.A. Ohl (Eds.),
Tsw12	nematodes	Fenoll et al (1997) In: Cellular and molecular aspects of plant-nematode interactions. Kluwer Academic, C. Fenoll, F.M.W. Grundler and S.A. Ohl (Eds.)
Hs1(pro1)	nematodes	WO 98/122335 - Jung
NsLTP	viral, fungal, bacterial	Molina & Garc´ia-Olmedo (1993) FEBS Lett, 316(2):119-22
RIP	viral, fungal	Tumer et al (1997) Proc Natl Acad Sci U S A, 94(8):3866-71

Examples of terminators particularly suitable for use in the gene constructs of the present invention include the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene terminator, the *Agrobacterium tumefaciens* octopine synthase (OCS) gene terminator sequence, the Cauliflower mosaic virus (CaMV) 35S gene terminator sequence, the *Oryza sativa* ADP-glucose pyrophosphorylase terminator sequence (t3'Bt2), the *Zea mays* zein gene terminator sequence, the *rbcs-1A* gene terminator, and the *rbcs-3A* gene terminator sequences, amongst others.

Preferred promoter sequences of the invention include root specific promoters and seed-specific promoters such as but not limited to the ones listed in Table 5, Table 4, and as outlined in the Examples.

Table 5. Exemplary root specific promoters for use in the performance of the present invention

NAME	ORIGIN	REFERENCE
SbPRP1	Soybean	Suzuki et al., Plant Mol Biol, 21: 109-119, 1993
636 bp fragment of TobRB7	Tobacco	Yamamoto et al., Plant Cell 3:371-382, 1991
GGPS3	Arabidopsis	Okada et al.,Plant Physiol 122: 1045-1056, 2000
580 bp fragment of prxEa	Arabidopsis	Wanapu and Shinmyo, Ann N Y Acad Sci 782: 107-114, 1996
Ids2 promoter	Barley	Okumura et al., Plant Mol Biol 25: 705-719, 1994
AtPRP3	Arabidopsis	Fowler et al., Plant Physiol 121: 1081-1092, 1999

Those skilled in the art will be aware of additional promoter sequences and terminator sequences which may be suitable for use in performing the invention. Such sequences may readily be used without any undue experimentation.

In the context of the current invention, "ectopic expression" or "ectopic overexpression" of a gene or a protein are conferring to expression patterns and/or expression levels of said gene or protein normally not occurring under natural conditions, more specifically is meant increased expression and/or increased expression levels. Ectopic expression can be achieved in a number of ways including operably linking of a coding sequence encoding said protein to an isolated homologous or heterologous promoter in order to create a chimeric gene and/or operably linking said coding sequence to its own isolated promoter (i.e. the unisolated promoter naturally driving expression of said protein) in order to create a recombinant gene duplication or gene multiplication effect. With "ectopic co-expression" is meant the ectopic expression or ectopic overexpression of two or more genes or proteins. The same or, more preferably, different promoters are used to confer ectopic expression of said genes or proteins.

Preferably, the promoter sequence used in the context of the present invention is operably linked to a coding sequence or open reading frame (ORF) encoding a cytokinin oxidase protein or a homologue, derivative or an immunologically active and/or functional fragment thereof as defined supra.

"Downregulation of expression" as used herein means lowering levels of gene expression and/or levels of active gene product and/or levels of gene product activity. Decreases in expression may be accomplished by e.g. the addition of coding sequences or parts thereof in a sense orientation (if resulting in co-suppression) or in an antisense orientation relative to a promoter sequence and furthermore by e.g. insertion mutagenesis (e.g. T-DNA insertion or transposon insertion) or by gene silencing strategies as described by e.g. Angell and Baulcombe (1998 - WO9836083), Lowe et al. (1989 - WO9853083), Lederer et al. (1999 - WO9915682) or Wang et al. (1999 - WO9953050). Genetic constructs aimed at silencing gene expression may have the nucleotide sequence of said gene (or one or more parts thereof) contained therein in a sense and/or antisense orientation relative to the promoter sequence. Another method to downregulate gene expression comprises the use of ribozymes.

Modulating, including lowering, the level of active gene products or of gene product activity can be achieved by administering or exposing cells, tissues, organs or organisms to said gene product, a homologue, derivative and/or immunologically active fragment thereof. Immunomodulation is another example of a technique capable of downregulation levels of active gene product and/or of gene product activity and comprises administration of or exposing to or expressing antibodies to said gene product to or in cells, tissues, organs or organisms wherein levels of said gene product and/or gene product activity are to be modulated. Such antibodies comprise "plantibodies", single chain antibodies, IgG antibodies and heavy chain camel antibodies as well as fragments thereof.

Modulating, including lowering, the level of active gene products or of gene product activity can furthermore be achieved by administering or exposing cells, tissues, organs or organisms to an agonist of said gene product or the activity thereof. Such agonists include proteins (comprising e.g. kinases and

proteinases) and chemical compounds identified according to the current invention as described supra.

In the context of the current invention is envisaged the downregulation of the expression of a cytokinin oxidase gene as defined earlier. Preferably said cytokinin oxidase gene is a plant cytokinin oxidase gene, more specifically an *AtCKX*. The invention further comprises downregulation of levels of a cytokinin oxidase protein or of a cytokinin oxidase activity whereby said cytokinin oxidase protein has been defined supra. Preferably said cytokinin oxidase protein is a plant cytokinin oxidase, more specifically an AtCKX.

By "modifying cell fate and/or plant development and/or plant morphology and/or biochemistry and/or physiology" is meant that one or more developmental and/or morphological and/or biochemical and/or physiological characteristics of a plant is altered by the performance of one or more steps pertaining to the invention described herein.

"Cell fate" refers to the cell-type or cellular characteristics of a particular cell that are produced during plant development or a cellular process therefor, in particular during the cell cycle or as a consequence of a cell cycle process.

"Plant development" or the term "plant developmental characteristic" or similar term shall, when used herein, be taken to mean any cellular process of a plant that is involved in determining the developmental fate of a plant cell, in particular the specific tissue or organ type into which a progenitor cell will develop. Cellular processes relevant to plant development will be known to those skilled in the art. Such processes include, for example, morphogenesis, photomorphogenesis, shoot development, root development, vegetative development, reproductive development, stem elongation, flowering, and regulatory mechanisms involved in determining cell fate, in particular a process or regulatory process involving the cell cycle.

"Plant morphology" or the term "plant morphological characteristic" or similar term will, when used herein, be understood by those skilled in the art to refer to the external appearance of a plant, including any one or more structural

features or combination of structural features thereof. Such structural features include the shape, size, number, position, color, texture, arrangement, and patternation of any cell, tissue or organ or groups of cells, tissues or organs of a plant, including the root, stem, leaf, shoot, petiole, trichome, flower, petal, stigma, style, stamen, pollen, ovule, seed, embryo, endosperm, seed coat, aleurone, fiber, fruit, cambium, wood, heartwood, parenchyma, aerenchyma, sieve element, phloem or vascular tissue, amongst others.

"Plant biochemistry" or the term "plant biochemical characteristic" or similar term will, when used herein, be understood by those skilled in the art to refer to the metabolic and catalytic processes of a plant, including primary and secondary metabolism and the products thereof, including any small molecules, macromolecules or chemical compounds, such as but not limited to starches, sugars, proteins, peptides, enzymes, hormones, growth factors, nucleic acid molecules, celluloses, hemicelluloses, calloses, lectins, fibers, pigments such as anthocyanins, vitamins, minerals, micronutrients, or macronutrients, that are produced by plants.

"Plant physiology" or the term "plant physiological characteristic" or similar term will, when used herein, be understood to refer to the functional processes of a plant, including developmental processes such as growth, expansion and differentiation, sexual development, sexual reproduction, seed set, seed development, grain filling, asexual reproduction, cell division, dormancy, germination, light adaptation, photosynthesis, leaf expansion, fiber production, secondary growth or wood production, amongst others; responses of a plant to externally-applied factors such as metals, chemicals, hormones, growth factors, environment and environmental stress factors (e.g. anoxia, hypoxia, high temperature, low temperature, dehydration, light, daylength, flooding, salt, heavy metals, amongst others), including adaptive responses of plants to said externally-applied factors.

Means for introducing recombinant DNA into plant tissue or cells include, but are not limited to, transformation using $CaCl_2$ and variations thereof, in particular the method described by Hanahan (1983), direct DNA uptake into

protoplasts (Krens *et al*, 1982; Paszkowski *et al*, 1984), PEG-mediated uptake to protoplasts (Armstrong *et al*, 1990) microparticle bombardment, electroporation (Fromm *et al.,* 1985), microinjection of DNA (Crossway *et al.*, 1986), microparticle bombardment of tissue explants or cells (Christou *et al*, 1988; Sanford, 1988), vacuum-infiltration of tissue with nucleic acid, or in the case of plants, T-DNA-mediated transfer from *Agrobacterium* to the plant tissue as described essentially by An *et al.*(1985), Dodds *et al.*, (1985), Herrera-Estrella *et al.* (1983a, 1983b, 1985). Methods for transformation of monocotyledonous plants are well known in the art and include *Agrobacterium*-mediated transformation (Cheng *et al.*, 1997 - WO9748814; Hansen 1998 - WO9854961; Hiei *et al.*, 1994 - WO9400977; Hiei *et al.*, 1998 - WO9817813; Rikiishi *et al.*, 1999 - WO9904618; Saito *et al.*, 1995 - WO9506722), microprojectile bombardment (Adams *et al.*, 1999 - US5969213; Bowen *et al.*, 1998 - US5736369; Chang *et al.*, 1994 - WO9413822; Lundquist *et al.*, 1999 - US5874265/US5990390; Vasil and Vasil, 1995 - US5405765. Walker *et al.*, 1999 - US5955362), DNA uptake (Eyal *et al.*, 1993 - WO9318168), microinjection of Agrobacterium cells (von Holt, 1994 - DE4309203) and sonication (Finer *et al.*, 1997 - US5693512).

For microparticle bombardment of cells, a microparticle is propelled into a cell to produce a transformed cell. Any suitable ballistic cell transformation methodology and apparatus can be used in performing the present invention. Exemplary apparatus and procedures are disclosed by Stomp *et al.* (U.S. Patent No. 5,122,466) and Sanford and Wolf (U.S. Patent No. 4,945,050). When using ballistic transformation procedures, the gene construct may incorporate a plasmid capable of replicating in the cell to be transformed. Examples of microparticles suitable for use in such systems include 1 to 5 μm gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

A whole plant may be regenerated from the transformed or transfected cell, in accordance with procedures well known in the art. Plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a gene construct of the present invention and a whole plant

regenerated therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

The term "organogenesis", as used herein, means a process by which shoots and roots are developed sequentially from meristematic centers.

The term "embryogenesis", as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes.

Preferably, the plant is produced according to the inventive method is transfected or transformed with a genetic sequence, or amenable to the introduction of a protein, by any art-recognized means, such as microprojectile bombardment, microinjection, *Agrobacterium*-mediated transformation (including *in planta* transformation), protoplast fusion, or electroporation, amongst others. Most preferably said plant is produced by *Agrobacterium*-mediated transformation.

Agrobacterium-mediated transformation or agrolistic transformation of plants, yeast, molds or filamentous fungi is based on the transfer of part of the transformation vector sequences, called the T-DNA, to the nucleus and on integration of said T-DNA in the genome of said eukaryote.

With "Agrobacterium" is meant a member of the Agrobacteriaceae, more preferably Agrobacterium or Rhizobacterium and most preferably Agrobacterium tumefaciens.

With "T-DNA", or transferred DNA, is meant that part of the transformation vector flanked by T-DNA borders which is, after activation of the *Agrobacterium vir* genes, nicked at the T-DNA borders and is transferred as a single stranded DNA to the nucleus of an eukaryotic cell.

When used herein, with "T-DNA borders", "T-DNA border region", or "border region" are meant either right T-DNA border (RB) or left T-DNA border (LB). Such a border comprises a core sequence flanked by a border inner region as part of the T-DNA flanking the border and/or a border outer region as part of the vector backbone flanking the border. The core sequences comprise 22 bp in case of octopine-type vectors and 25 bp in case of nopaline-type vectors. The core sequences in the right border region and left border region form imperfect repeats. Border core sequences are indispensable for recognition and processing by the *Agrobacterium* nicking complex consisting of at least VirD1 and VirD2. Core sequences flanking a T-DNA are sufficient to promote transfer of said T-DNA. However, efficiency of transformation using transformation vectors carrying said T-DNA solely flanked by said core sequences is low. Border inner and outer regions are known to modulate efficiency of T-DNA transfer (Wang et al. 1987). One element enhancing T-DNA transfer has been characterized and resides in the right border outer region and is called *overdrive* (Peralta et al. 1986, van Haaren et al. 1987).

With "T-DNA transformation vector" or "T-DNA vector" is meant any vector encompassing a T-DNA sequence flanked by a right and left T-DNA border consisting of at least the right and left border core sequences, respectively, and used for transformation of any eukaryotic cell.

With "T-DNA vector backbone sequence" or "T-DNA vector backbone sequences" is meant all DNA of a T-DNA containing vector that lies outside of the T-DNA borders and, more specifically, outside the nicking sites of the border core imperfect repeats.

The current invention includes optimized T-DNA vectors such that vector backbone integration in the genome of a eukaryotic cell is minimized or absent. With "optimized T-DNA vector" is meant a T-DNA vector designed either to decrease or abolish transfer of vector backbone sequences to the genome of a eukaryotic cell. Such T-DNA vectors are known to the one familiar with the art and include those described by Hanson et al. (1999) and by Stuiver et al. (1999 - WO9901563).

The current invention clearly considers the inclusion of a DNA sequence encoding a cytokinin oxidase, homologue, derivative or immunologically active and/or functional fragment thereof as defined supra, in any T-DNA vector comprising binary transformation vectors, super-binary transformation vectors, co-integrate transformation vectors, Ri-derived transformation vectors as well as in T-DNA carrying vectors used in agrolistic transformation. Preferably, said cytokinin oxidase is a plant cytokinin oxidase, more specifically an *Arabidopsis thaliana* (At)CKX.

With "binary transformation vector" is meant a T-DNA transformation vector comprising:

(a) a T-DNA region comprising at least one gene of interest and/or at least one selectable marker active in the eukaryotic cell to be transformed; and

(b) a vector backbone region comprising at least origins of replication active in *E. coli* and *Agrobacterium* and markers for selection in *E. coli* and *Agrobacterium*.

The T-DNA borders of a binary transformation vector can be derived from octopine-type or nopaline-type Ti plasmids or from both. The T-DNA of a binary vector is only transferred to a eukaryotic cell in conjunction with a helper plasmid.

With "helper plasmid" is meant a plasmid that is stably maintained in *Agrobacterium* and is at least carrying the set of *vir* genes necessary for enabling transfer of the T-DNA. Said set of *vir* genes can be derived from either octopine-type or nopaline-type Ti plasmids or from both.

With "super-binary transformation vector" is meant a binary transformation vector additionally carrying in the vector backbone region a *vir* region of the Ti plasmid pTiBo542 of the super-virulent *A. tumefaciens* strain A281 (EP0604662, EP0687730). Super-binary transformation vectors are used in conjunction with a helper plasmid.

With "co-integrate transformation vector" is meant a T-DNA vector at least comprising:

(a) a T-DNA region comprising at least one gene of interest and/or at least one selectable marker active in plants; and

(b) a vector backbone region comprising at least origins of replication active in *Escherichia coli* and *Agrobacterium,* and markers for selection in *E. coli* and *Agrobacterium,* and a set of *vir* genes necessary for enabling transfer of the T-DNA.

The T-DNA borders and said set of *vir* genes of a said T-DNA vector can be derived from either octopine-type or nopaline-type Ti plasmids or from both.

With "Ri-derived plant transformation vector" is meant a binary transformation vector in which the T-DNA borders are derived from a Ti plasmid and said binary transformation vector being used in conjunction with a 'helper' Ri-plasmid carrying the necessary set of *vir* genes.

As used herein, the term "selectable marker gene" or "selectable marker" or "marker for selection" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a gene construct of the invention or a derivative thereof. Suitable selectable marker genes contemplated herein include the ampicillin resistance (Amp^r), tetracycline resistance gene (Tc^r), bacterial kanamycin resistance gene (Kan^r), phosphinothricin resistance gene, neomycin phosphotransferase gene (*npt*II), hygromycin resistance gene, β-glucuronidase (GUS) gene, chloramphenicol acetyltransferase (CAT) gene, green fluorescent protein (*gfp*) gene (Haseloff *et al,* 1997), and luciferase gene, amongst others.

With "agrolistics", "agrolistic transformation" or "agrolistic transfer" is meant here a transformation method combining features of *Agrobacterium*-mediated transformation and of biolistic DNA delivery. As such, a T-DNA containing target plasmid is co-delivered with DNA/RNA enabling in planta production of VirD1 and VirD2 with or without VirE2 (Hansen and Chilton 1996; Hansen et al. 1997; Hansen and Chilton 1997 - WO9712046).

With "foreign DNA" is meant any DNA sequence that is introduced in the host's genome by recombinant techniques. Said foreign DNA includes e.g. a T-DNA sequence or a part thereof such as the T-DNA sequence comprising the selectable marker in an expressible format. Foreign DNA furthermore include intervening DNA sequences as defined supra.

With "recombination event" is meant either a site-specific recombination event or a recombination event effected by transposon 'jumping'.

With "recombinase" is meant either a site-specific recombinase or a transposase.

With "recombination site" is meant either site-specific recombination sites or transposon border sequences.

With "site specific recombination event" is meant an event catalyzed by a system generally consisting of three elements: a pair of DNA sequences (the site-specific recombination sequences or sites) and a specific enzyme (the site-specific recombinase). The site-specific recombinase catalyzes a recombination reaction only between two site-specific recombination sequences depending on the orientation of the site-specific recombination sequences. Sequences intervening between two site-specific recombination sites will be inverted in the presence of the site-specific recombinase when the site-specific recombination sequences are oriented in opposite directions relative to one another (i.e. inverted repeats). If the site-specific recombination sequences are oriented in the same direction relative to one another (i.e. direct repeats), then any intervening sequences will be deleted upon interaction with the site-specific recombinase. Thus, if the site-specific recombination sequences are present as direct repeats at both ends of a foreign DNA sequence integrated into a eukaryotic genome, such integration of said sequences can subsequently be reversed by interaction of the site-specific recombination sequences with the corresponding site specific recombinase.

A number of different site specific recombinase systems can be used including but not limited to the Cre/lox system of bacteriophage P1, the FLP/FRT system of yeast, the Gin recombinase of phage Mu, the Pin recombinase of *E. coli*,

the PinB, PinD and PinF from *Shigella*, and the R/RS system of the pSR1 plasmid. Recombinases generally are integrases, resolvases or flippases. Also dual-specific recombinases can be used in conjunction with direct or indirect repeats of two different site-specific recombination sites corresponding to the dual-specific recombinase (WO99/25840). The two preferred site-specific recombinase systems are the bacteriophage P1 Cre/lox and the yeast FLP/FRT systems. In these systems a recombinase (Cre or FLP) interact specifically with its respective site-specific recombination sequence (lox or FRT respectively) to invert or excise the intervening sequences. The site-specific recombination sequences for each of these two systems are relatively short (34 bp for lox and 47 bp for FRT). Some of these systems have already been used with high efficiency in plants such as tobacco (Dale et al. 1990) and *Arabidopsis* (Osborne et al. 1995). Site-specific recombination systems have many applications in plant molecular biology including methods for control of homologous recombination (e.g. US5527695), for targeted insertion, gene stacking, etc. (WO99/25821) and for resolution of complex T-DNA integration patterns or for excision of a selectable marker (WO99/23202).

Although the site-specific recombination sequences must be linked to the ends of the DNA to be excised or to be inverted, the gene encoding the site specific recombinase may be located elsewhere. For example, the recombinase gene could already be present in the eukaryote's DNA or could be supplied by a later introduced DNA fragment either introduced directly into cells, through crossing or through cross-pollination. Alternatively, a substantially purified recombinase protein could be introduced directly into the eukaryotic cell, e.g. by micro-injection or particle bombardment. Typically, the site-specific recombinase coding region will be operably linked to regulatory sequences enabling expression of the site-specific recombinase in the eukaryotic cell.

With "recombination event effected by transposon jumping" or "transposase-mediated recombination" is meant a recombination event catalyzed by a system consisting of three elements: a pair of DNA sequences (the transposon border sequences) and a specific enzyme (the transposase). The

transposase catalyzes a recombination reaction only between two transposon border sequences which are arranged as inverted repeats.

A number of different transposon/transposase systems can be used including but not limited to the Ds/Ac system, the Spm system and the Mu system. These systems originate from corn but it has been shown that at least the Ds/Ac and the Spm system also function in other plants (Fedoroff et al. 1993, Schlappi et al. 1993, Van Sluys et al. 1987). Preferred are the Ds- and the Spm-type transposons which are delineated by 11 bp- and 13 bp- border sequences, respectively.

Although the transposon border sequences must be linked to the ends of the DNA to be excised, the gene encoding the transposase may be located elsewhere. For example, the recombinase gene could already be present in the eukaryote's DNA or could be supplied by a later introduced DNA fragment either introduced directly into cells, through crossing or through cross-pollination. Alternatively, a substantially purified transposase protein could be introduced directly into cells, e.g. by microinjection or by particle bombardment.

As part of the current invention, transposon border sequences are included in a foreign DNA sequence such that they lie outside said DNA sequence and transform said DNA into a transposon-like entity that can move by the action of a transposase.

As transposons often reintegrate at another locus of the host's genome, segregation of the progeny of the hosts in which the transposase was allowed to act might be necessary to separate transformed hosts containing e.g. only the transposon footprint and transformed hosts still containing the foreign DNA.

In performing the present invention, the genetic element is preferably induced to mobilize, such as, for example, by the expression of a recombinase protein in the cell which contacts the integration site of the genetic element and facilitates a recombination event therein, excising the genetic element completely, or alternatively, leaving a "footprint", generally of about 20 nucleotides in length or greater, at the original integration site. Those hosts and host parts that have

been produced according to the inventive method can be identified by standard nucleic acid hybridization and/or amplification techniques to detect the presence of the mobilizable genetic element or a gene construct comprising the same. Alternatively, in the case of transformed host cells, tissues, and hosts wherein the mobilizable genetic element has been excised, it is possible to detect a footprint in the genome of the host which has been left following the excision event, using such techniques. As used herein, the term "footprint" shall be taken to refer to any derivative of a mobilizable genetic element or gene construct comprising the same as described herein which is produced by excision, deletion or other removal of the mobilizable genetic element from the genome of a cell transformed previously with said gene construct. A footprint generally comprises at least a single copy of the recombination loci or transposon used to promote excision. However, a footprint may comprise additional sequences derived from the gene construct, for example nucleotide sequences derived from the left border sequence, right border sequence, origin of replication, recombinase-encoding or transposase-encoding sequence if used, or other vector-derived nucleotide sequences. Accordingly, a footprint is identifiable according to the nucleotide sequence of the recombination locus or transposon of the gene construct used, such as, for example, a sequence of nucleotides corresponding or complementary to a *lox* site or *frt* site.

The term “cell cycle” means the cyclic biochemical and structural events associated with growth and with division of cells, and in particular with the regulation of the replication of DNA and mitosis. Cell cycle includes phases called: G0, Gap1 (G1), DNA synthesis (S), Gap2 (G2), and mitosis (M). Normally these four phases occur sequentially, however, the cell cycle also includes modified cycles wherein one or more phases are absent resulting in modified cell cycle such as endomitosis, acytokinesis, polyploidy, polyteny, and endoreduplication.

The term "cell cycle progression" refers to the process of passing through the different cell cycle phases. The term "cell cycle progression rate" accordingly refers to the speed at which said cell cycle phases are run through or the time spans required to complete said cell cycle phases.

With "two-hybrid assay" is meant an assay that is based on the observation that many eukaryotic transcription factors comprise two domains, a DNA-binding domain (DB) and an activation domain (AD) which, when physically separated (i.e. disruption of the covalent linkage) do not effectuate target gene expression. Two proteins able to interact physically with one of said proteins fused to DB and the other of said proteins fused to AD will re-unite the DB and AD domains of the transcription factor resulting in target gene expression. The target gene in the yeast two-hybrid assay is usually a reporter gene such as the β-galactosidase gene. Interaction between protein partners in the yeast two-hybrid assay can thus be quantified by measuring the activity of the reporter gene product (Bartel and Fields 1997). Alternatively, a mammalian two-hybrid system can be used which includes e.g. a chimeric green fluorescent protein encoding reporter gene (Shioda *et al.*, 2000).

Furthermore, folding simulations and computer redesign of structural motifs of the protein of the invention can be performed using appropriate computer programs (Olszewski, Proteins 25 (1996), 286-299; Hoffman, Comput. Appl. Biosci. 1 (1995), 675-679). Computer modeling of protein folding can be used for the conformational and energetic analysis of detailed peptide and protein models (Monge, J. Mol. Biol. 247 (1995), 995-1012; Renouf, Adv. Exp. Med. Biol. 376 (1995), 37-45). In particular, the appropriate programs can be used for the identification of interactive sites of the cytokinin oxidases, its ligands or other interacting proteins by computer assistant searches for complementary peptide sequences (Fassina, Immunomethods 5 (1994), 114-120). Further appropriate computer systems for the design of protein and peptides are described in the prior art, for example in Berry, Biochem. Soc. Trans. 22 (1994), 1033-1036; Wodak, Ann, N. Y. Acac. Sci. 501 (1987), 1-13; Pabo, Biochemistry 25 (1986), 5987-5991. The results obtained form the above-described computer analysis can be used for, e.g. the preparation of peptidomimetics of the protein of the invention or fragments thereof. Such pseudopeptide analogues of the natural amino acid sequence of the protein may very efficiently mimic the parent protein (Benkirane, J. Biol. Chem. 271 (1996), 33218-33224). For example, incorporation of easily available achiral Ω-amino acid residues into a protein of the invention or a

fragment thereof results in the substitution of amino bonds by polymethylene units of an aliphatic chain, thereby providing a convenient strategy for constructing a peptidomimetic (Banerjee, Biopolymers 39 (1996), 769-777). Superactive peptidomimetic analogues of small peptide hormones in other systems are described in the prior art (Zhang, Biochem. Biophys. Res. Commun. 224 (1996), 327-331). Appropriate peptidomimetics of the protein of the present invention can also be identified by the synthesis of peptidomimetic combinatorial libraries through successive amine alkylation and testing the resulting compounds, e.g., for their binding, kinase inhibitory and/or immunological properties. Methods for the generation and use of peptidomimetic combinatorial libraries are described in the prior art, for example in Ostresh, Methods in Enzymology 267 (1996), 220-234 and Dorner, Bioorg. Med. Chem. 4 (1996), 709-715.

Furthermore, a three-dimensional and/or crystallographic structure of the protein of the invention can be used for the design of peptidomimetic inhibitors of the biological activity of the protein of the invention (Rose, Biochemistry 35 (1996), 12933-12944; Ruterber, Bioorg. Med. Chem. 4 (1996), 1545-1558).

The compounds to be obtained or identified in the methods of the invention can be compounds that are able to bind to any of the nucleic acids, peptides or proteins of the invention. Other interesting compounds to be identified are compounds that modulate the expression of the genes or the proteins of the invention in such a way that either the expression of said gene or protein is enhanced or decreased by the action of said compound. Alternatively the compound can exert his action by enhancing or decreasing the activity of any of the proteins of the invention. Herein, preferred proteins are novel cytokinin oxidases.

Said compound or plurality of compounds may be comprised in, for example, samples, e.g., cell extracts from, e.g., plants, animals or microorganisms. Furthermore, said compound(s) may be known in the art but hitherto not known to be capable of suppressing or activating cytokinin oxidase interacting proteins. The reaction mixture may be a cell free extract of may comprise a cell or tissue culture. Suitable set ups for the method of the invention are known to the person

skilled in the art and are, for example, generally described in Alberts et al., Molecular Biology of the Cell, third edition (1994), in particular Chapter 17. The plurality of compounds may be, e.g., added to the reaction mixture, culture medium or injected into the cell.

If a sample containing a compound or a plurality of compounds is identified in the method of the invention, then it is either possible to isolate the compound form the original sample identified as containing the compound capable of acting as an agonist, or one can further subdivide the original sample, for example, if it consists of a plurality of different compounds, so as to reduce the number of different substances per sample and repeat the method with the subdivisions of the original sample. Depending on the complexity of the samples, the steps described above can be performed several times, preferably until the sample identified according to the method of the invention only comprises a limited number of or only one substance(s). Preferably said sample comprises substances or similar chemical and/or physical properties, and most preferably said substances are identical. Preferably, the compound identified according to the above-described method or its derivative is further formulated in a form suitable for the application in plant breeding or plant cell and tissue culture.

The term “early vigor” refers to the ability of a plant to grow rapidly during early development, and relates to the successful establishment, after germination, of a well-developed root system and a well-developed photosynthetic apparatus.

The term “resistance to lodging” or “standability” refers to the ability of a plant to fix itself to the soil. For plants with an erect or semi-erect growth habit this term also refers to the ability to maintain an upright position under adverse (environmental) conditions. This trait relates to the size, depth and morphology of the root system.

The term ‘grafting’ as used herein, refers to the joining together of the parts of two different plants so that they bind together and the sap can flow, thus forming a single new plant that can grow and develop. A graft therefore consists

of two parts: (i) the lower part is the rootstock as referred to herein and essentially consists of the root system and a portion of the stem, and (ii) the upper part, the scion or graft, which gives rise to the aerial parts of the plant.

As used herein, tblastn refers to an alignment tool that is part of the BLAST (Basic Local Alignment Search Tool) family of programs (http://www.ncbi.nlm.nih.gov/BLAST/). BLAST aims to identify regions of optimal local alignment, i.e. the alignment of some portion of two nucleic acid or protein sequences, to detect relationships among sequences which share only isolated regions of similarity (Altschul et al., 1990). In the present invention, tblastn of the BLAST 2.0 suite of programs was used to compare the maize cytokinin oxidase protein sequence against a nucleotide sequence database dynamically translated in all reading frames (Altschul et al., Nucleic Acids Res. 25: 3389-3402 (1997)).

The following examples are given by means of illustration of the present invention and are in no way limiting. The contents of all references included in this application are incorporated by reference herein as if fully set forth.

EXAMPLES

Example 1. Brief description of the sequences of the invention

SEQ ID NO:	DESCRIPTION
1	*AtCKX1* genomic
2	*AtCKX1* protein
3	*AtCKX2* genomic
4	*AtCKX2* protein
5	*AtCKX3* genomic
6	*AtCKX3* protein
7	*AtCKX4* genomic
8	*AtCKX4* protein
9	*AtCKX5* genomic (short version)
10	*AtCKX5* protein (short version)
11	*AtCKX6* genomic
12	*AtCKX6* protein
13	5'primer *AtCKX1*
14	3'primer *AtCKX1*
15	5'primer *AtCKX2*
16	3'primer *AtCKX2*
17	5'primer *AtCKX3*
18	3'primer *AtCKX3*
19	5'primer *AtCKX4*
20	3'primer *AtCKX4*
21	5'primer *AtCKX5*
22	3'primer *AtCKX5*

23	5'primer *AtCKX6*
24	3'primer *AtCKX6*
25	*AtCKX1* cDNA
26	*AtCKX2* cDNA
27	*AtCKX3* cDNA
28	*AtCKX4* cDNA
29	*AtCKX5* cDNA (short version)
30	*AtCKX6* cDNA
31	*AtCKX2* cDNA fragment
32	*AtCKX2* peptide fragment
33	*AtCKX5* genomic (long version)
34	*AtCKX5* cDNA (long version)
35	*AtCKX5* protein (long version)
36	root clavata homolog promoter

<u>Example 2. Identification of candidate cytokinin oxidase encoding genes from *Arabidopsis thaliana*</u>

Six different genes were identified from *Arabidopsis thaliana* that bear sequence similarity to a cytokinin oxidase gene from maize (Morris *et al.*, Biochem Biophys Res Comm 255:328-333, 1999; Houda-Herin *et al.* Plant J 17:615-626; WO 99/06571). These genes were found by screening 6-frame translations of nucleotide sequences from public genomic databases with the maize protein sequence, employing tblastn program. These sequences were designated as *Arabidopsis thaliana* cytokinin oxidase-like genes or *AtCKX*. They were arbitrarily numbered as *AtCKX1 to AtCKX6*. The below list summarizes the information on these genes. The predicted ORF borders and protein sequences are indicative, in order to illustrate by approximation the protein sequence divergence between the Arabidopsis and maize cytokinin oxidases, as well as amongst the different Arabidopsis cytokinin oxidases. The ORF borders and protein sequences shown should not be taken as conclusive evidence for the mode of action of these *AtCKX* genes. For DNA and protein sequence comparisons the program MegAlign from DNAstar was used. This program uses the Clustal method for alignments. For multiple alignments of protein and cDNA sequences the gap penalty and gap length penalty was set at 10 each. For pairwise alignments of proteins the parameters were as follows: Ktuple at 1; Gap penalty at 3; window at 5; diagonals saved at 5. For pairwise alignments of cDNA's the parameters were as follows: Ktuple at 2; Gap penalty at 5; window at 4; diagonals saved at 4. The similarity groups for protein alignments was: (M,I,L,V), (F,W,Y), (G,A), (S,T), (R,K,H), (E,D), (N,Q). The values that are indicated amongst the Arabidopsis cDNA and protein sequences represent the lowest and highest values found with all combinations.

<u>A. Gene name:</u> ***<u>AtCKX1</u>*** (*Arabidopsis thaliana* cytokinin oxidase-like protein 1, SEQ ID NO: 1)

Location in database (accession number, location on bac): AC002510, *Arabidopsis thaliana* chromosome II section 225 of 255 of the complete sequence. Sequence from clones T32G6.

ORF predicted in the database:

15517..16183, 16415..16542, 16631..16891, 16995..17257, 17344..17752

The *AtCKX1* cDNA sequence is listed as SEQ ID NO: 25

Predicted protein sequence: SEQ ID NO: 2:

Homologies

% identity with *Z. mays* cDNA:

31.5% (Dnastar/MegAlign - Clustal method)

% similarity with *Z. mays* protein:

32.2% (Dnastar/MegAlign - Clustal method)

% identity with other *Arabidopsis* cDNA's (range):

38.2% (*AtCKX2*) – 54.1% (*AtCKX6*) (Dnastar/MegAlign - Clustal method)

% similarity with other *Arabidopsis* proteins (range):

37.1% (*AtCKX2*) – 58.1% (*AtCKX6*) (Dnastar/MegAlign - Clustal method)

B. Gene name: *AtCKX2* (*Arabidopsis thaliana* cytokinin oxidase-like protein 2, SEQ ID NO: 3)

Location in database (accession number, location on bac): AC005917, *Arabidopsis thaliana* chromosome II section 113 of 255 of the complete sequence. Sequence from clones F27F23, F3P11.

ORF predicted in the database:

complement, 40721..41012, 41054..41364, 41513..41770, 42535..42662, 43153..43711

Please note: The cDNA sequence identified by the inventor using the gene prediction program NetPlantGene (http://www.cbs.dtu.dk/services/NetGene2/) was different than the one annotated in the database. Based on the new cDNA sequence the ORF predicted in the database was revised:

complement, 40721..41012, 41095..41364, 41513..41770, 42535..42662, 43153..43711

The protein sequence encoded by this cDNA is listed as SEQ ID NO: 4. The cDNA of *AtCKX2* was cloned by RT-PCR from total RNA of *AtCKX2* transgenic plant tissue with the one-step RT-PCR kit (Qiagen, Hilden, Germany) and sequenced using an ABI PRISM Big Dye Terminator cycle sequencing reaction kit (Perkin Elmer Applied Biosystems Division). This confirmed that the cDNA sequence identified and predicted by the inventor was correct. The new *AtCKX2* cDNA sequence is listed as SEQ ID NO: 26. An 84-bp fragment corresponding to nucleotides 1171 through 1254 of the *AtCKX2* cDNA is listed as SEQ ID NO: 31. The corresponding peptide sequence of this 84-bp cDNA sequence is listed as SEQ ID NO: 32.

Homologies

% identity with *Z. mays* cDNA:

38.4% (Dnastar/MegAlign - Clustal method)

% similarity with *Z. mays* protein:

37.5% (Dnastar/MegAlign - Clustal method)

% identity with other *Arabidopsis* cDNA's (range):

34.9% (*AtCKX6*) – 64.5% (*AtCKX4*) (Dnastar/MegAlign - Clustal method)

% similarity with other *Arabidopsis* proteins (range):

36.5% (*AtCKX6*) – 66.1% (*AtCKX4*) (Dnastar/MegAlign - Clustal method)

C. Gene name: *AtCKX3* (*Arabidopsis thaliana* cytokinin oxidase-like protein 3, SEQ ID NO: 5)

Location in database (accession number, location on bac): AB024035, *Arabidopsis thaliana* genomic DNA, chromosome 5, P1 clone: MHM17, complete sequence.

No prediction of the ORF in the database.

The gene was identified by the inventor using several gene prediction programs including GRAIL (ftp: //arthur.epm.ornl.gov/pub/xgrail), Genscan (http://CCR-081.mit.edu/GENSCAN html) and NetPlantGene (http://www.cbs.dtu.dk/services/NetGene2/):

complement, 29415..29718, 29813..30081, 30183..30443, 30529..30656, 32107..32716

The new *AtCKX3* cDNA sequence identified by the inventor is listed as SEQ ID NO: 27

Predicted protein sequence, based on own ORF prediction: SEQ ID NO: 6

Homologies

% identity with *Z. mays* cDNA:

38.7% (Dnastar/MegAlign - Clustal method)

% similarity with *Z. mays* protein:

39.2% (Dnastar/MegAlign - Clustal method)

% identity with other *Arabidopsis* cDNA's (range):

38.8% (*AtCKX6*) – 51.0% (*AtCKX2*) (Dnastar/MegAlign - Clustal method)

% similarity with other *Arabidopsis* proteins (range):

39.9% (*AtCKX6*) – 46.7% (*AtCKX2*) (Dnastar/MegAlign - Clustal method)

__D. Gene name: *AtCKX4*__ (*Arabidopsis thaliana* cytokinin oxidase-like protein 4, SEQ ID NO: 7)

Location in database (accession number, location on bac):

1) AL079344, *Arabidopsis thaliana* DNA chromosome 4, BAC clone T16L4 (ESSA project)

2) AL161575, *Arabidopsis thaliana* DNA chromosome 4, contig fragment No. 71.

ORF predicted in the database:

1) 76187..76814, 77189..77316, 77823..78080, 78318..78586, 78677..78968

2) 101002..101629, 102004..102131, 102638..102895, 103133..103401, 103492..103783

The *AtCKX4* cDNA sequence is listed as SEQ ID NO: 28

Predicted protein sequence: SEQ ID NO: 8

Homologies

% identity with *Z. mays* cDNA:

41.0% (Dnastar/MegAlign - Clustal method)

% similarity with *Z. mays* protein:

41.0% (Dnastar/MegAlign - Clustal method)

% identity with other *Arabidopsis* cDNA's (range):

35.2% (*AtCKX6*) – 64.5% (*AtCKX2*) (Dnastar/MegAlign - Clustal method)

% similarity with other *Arabidopsis* proteins (range):

35.1% (*AtCKX6*) – 66.1% (*AtCKX2*) (Dnastar/MegAlign - Clustal method)

E. Gene name: ***AtCKX5*** (*Arabidopsis thaliana* cytokinin oxidase-like protein 5, SEQ ID NO: 9)

Location in database (accession number, location on bac): AC023754, F1B16, complete sequence, chromosome 1

No prediction of the ORF in the database.

The gene was identified by the inventors using several gene prediction programs including GRAIL (ftp://arthur.epm.ornl.gov/pub/xgrail), Genscan (http://CCR-081.mit.edu/GEN SCAN.html) and NetPlantGene (http://www.cbs.dtu.dk/services/NetGene2/).

43756..44347, 44435..44562, 44700..44966, 45493..45755, 46200..46560

The new *AtCKX5* cDNA sequence identified and predicted by the inventor is listed as SEQ ID NO: 29. The predicted protein sequence for this cDNA is listed as SEQ ID NO: 10. A second potential ATG start codon is present 9 nucleotides more upstream in the genomic sequence. It is unclear which of these 2 start codons encodes the first amino acid of the protein. Therefore, a second potential *AtCKX5* cDNA starting at this upstream start codon is also listed in this invention as SEQ ID NO: 34. The corresponding genomic sequence is listed as SEQ ID NO: 33 and the encoded protein as SEQ ID NO: 35.

Homologies

% identity with *Z. mays* cDNA:

39.1% (Dnastar/MegAlign - Clustal method)

% similarity with *Z. mays* protein:

36.6% (Dnastar/MegAlign - Clustal method)

% identity with other *Arabidopsis* cDNA's (range):

40.1% (*AtCKX2*) – 44.0% (*AtCKX3*) (Dnastar/MegAlign - Clustal method)

% similarity with other *Arabidopsis* proteins (range):

41.6% (*AtCKX4*) – 46.4% (*AtCKX6*) (Dnastar/MegAlign - Clustal method)

F. Gene name: *AtCKX6* (*Arabidopsis thaliana* cytokinin oxidase-like protein 6, SEQ ID NO: 11)

Location in database (accession number, location on bac): AL163818, *Arabidopsis thaliana* DNA chromosome 3, P1 clone MAA21 (ESSA project).

ORF predicted in the database:

46630..47215, 47343..47470, 47591..47806, 47899..48161, 48244..48565

The *AtCKX6* cDNA sequence is listed as SEQ ID NO: 30

Predicted protein sequence: SEQ ID NO: 12

Homologies

% identity with *Z. mays* cDNA:

37.3% (Dnastar/MegAlign - Clustal method)

% similarity with *Z. mays* protein:

36.1% (Dnastar/MegAlign - Clustal method)

% identity with other *Arabidopsis* cDNA's (range):

34.9% (*AtCKX2*) – 54.1% (*AtCKX1*) (Dnastar/MegAlign - Clustal method)

% similarity with other *Arabidopsis* proteins (range):

35.1% (*AtCKX4*) – 58.1% (*AtCKX1*) (Dnastar/MegAlign - Clustal method)

Genes *AtCKX3* and *AtCKX5* were not annotated as putative cytokinin oxidases in the database and ORFs for these genes were not given. Furthermore, the ORF (and consequently the protein structures) predicted for *AtCKX2* was different from our own prediction and our prediction was confirmed by sequencing the *AtCKX2* cDNA.

A comparison of the gene structure of the *Arabidopsis AtCKX* genes 1 to 4 and the maize *CKX* gene is shown in Fig 1.

The predicted proteins encoded by the *Arabidopsis AtCKX* genes show between 32% and 41% sequence similarity with the maize protein, while they show between 35% and 66% sequence similarity to each other. Because of this reduced sequence conservation, it is not clear *a priori* whether the *Arabidopsis AtCKX* genes encode proteins with cytokinin oxidase activity. An alignment of the *Arabidopsis AtCKX* predicted proteins 1 to 4 and the maize *CKX* gene is shown in Fig 2.

Example 3. Transgenic plants overexpressing *AtCKX1* showed increased cytokinin oxidase activity and altered plant morphology

1. Description of the cloning process

The following primers were used to PCR amplify the *AtCKX1* gene from *Arabidopsis thaliana*, accession Columbia (non-homologous sequences used for cloning are in lower case):

Sequence of 5´ primer: cggtcgacATGGGATTGACCTCATCCTTACG (SEQ ID NO:13)

Sequence of 3´ primer: gcgtcgacTTATACAGTTCTAGGTTTCGGCAGTAT (SEQ ID NO: 14)

A 2235-bp PCR fragment, amplified by these primers, was inserted in the Sal I site of pUC19. The insert was sequenced and confirmed that the PCR amplification product did not contain any mutations. The SalI/SalI fragment of this vector was subcloned in the SalI site downstream of a modified CaMV 35S promoter (carrying three tetracycline operator sequences) in the binary vector pBinHyg-Tx (Gatz *et al.*, 1992). The resulting construct was introduced into tobacco and *Arabidopsis thaliana* through *Agrobacterium*-mediated transformation, using standard transformation protocols.

2. Molecular analysis of the transgenic lines

Several transgenic lines were identified that synthesize the *AtCKX1* transcript at high levels (Fig 3). Transgenic lines expressing *AtCKX1* transcript also showed increased cytokinin oxidase activity as determined by a standard assay for cytokinin oxidase activity based on conversion of [2-^{3}H]iP to adenine as described (Motyka et al., 1996). This is exemplified for 2 tobacco and 2 A*rabidopsis* lines in Table 6. This result proves that the *AtCKX1* gene encodes a protein with cytokinin oxidase activity.

Table 6. Cytokinin oxidase activity in *AtCKX1* transgenic plant tissues

Leaf sample		
Plant species	**Plant line**	**Cytokinin oxidase activity (nmol Ade/mg protein.h)**
Arabidopsis	Col-0 wild-type	0.009
	CKX1-11	0.024
	CKX1-22	0.026
	CKX1-22	0.027
Tobacco	SNN wild-type	0.004
	CKX1-SNN-8	0.016
	CKX1-SNN-28	0.021

3. Phenotypic description of the transgenic lines

3.1 In tobacco:

The plants had a dwarfed phenotype with reduced apical dominance (Figure 7 A, B and C) and increased root production (Figure 8).

Five categories of phenotype:

1) strong - 2 clones
2) intermediate - 3 clones
3) weak - 4 clones
4) tall plants (as WT) with large inflorescence - 5 clones
5) similar to WT, 9 clones

Height (see Fig. 7 B and C)

- WT: between 100-150 cm
- weak: approximately 75 cm
- intermediate: appr. 40-45 cm (main stem app. 25 cm but overgrown by side branches.
- strong: appr. 10 cm

The transgenics *AtCKX1-48* and *AtCKX1-50* displayed a strong phenotype. Below are measurements for stem elongation as compared to WT plants:

Line	Wild-type	AtCKX1-48	AtCKX1-50
Days after germination	Height (cm)	Height (cm)	Height (cm)
47	9.5 ± 0.5	1.3 ± 0.3	1.2 ± 0.2
58	22.4 ± 2.3	2.2 ±0.3	2.3 ± 0.3
68	35.3 ± 2.6	3.1 ± 0.5	2.6 ± 0.5
100	113.3 ± 9.8	7.1 ± 0.8	4.8 ± 0.9
117	138.6 ± 8.1	8.7 ± 0.7	6.6 ± 0.9
131	139.0 ± 9.3	9.3 ± 0.7	8.6 ± 1.0
152	136.6 ± 10.4	10.9 ± 1.1	10.0 ± 1.0
165		11.8 ± 1.9	11.4 ± 1.4
181		16.5 ± 1.7	14.9 ± 1.2
198		19.5 ± 1.5	18.1 ± 1.3

Experimental: Plants were grown in soil in a greenhouse. Data were collected from at least ten plants per line.

Leaves (see Figure 7 D and E)

The shape of leaves of *AtCKX1* transgenic expressors was lanceolate (longer and narrow): the width-to-length ratio of mature leaves was reduced from 1:2 in wild type plants to 1:3 in *AtCKX1* transgenics (Figure 7 E). The number of leaves and leaf surface was reduced compared to WT (see Figure 7 D). A prominent difference was also noted for progression of leaf senescence. In WT tobacco, leaf senescence starts in the most basal leaves and leads to a uniform reduction of leaf pigment (Figure 7 E). By contrast, ageing leaves of strongly expressing *AtCKX1* plants stayed green along the leaf veins and turned yellow in the intercostal regions, indicating altered leaf senescence. The texture of older leaves was more rigid.

Roots

In vitro grown plants highly expressing the gene were easily distinguishable from the WT by their ability to form more roots which are thicker (stronger) (Figure 8 A), as well as by forming aerial roots along the stem.

The primary root was longer and the number of lateral and adventitious roots was higher as illustrated in Figure 8 C for *AtCKX1-50* overexpressing seedlings (see also Example 9).

The dose-response curve of root growth inhibition by exogenous cytokinin showed that roots of transgenic seedlings are more cytokinin resistant than WT roots (Figure 8 D). The resistance of *AtCKX1* transgenics to iPR was less marked than for *AtCKX2*, which is consistent with the smaller changes in iP-type cytokinins in the latter (see Table 10).

A large increase in root biomass was observed for adult plants grown in soil (see Figure 8 B for a plant grown in soil for 4 to 5 months) despite the fact that growth of the aerial plant parts was highly reduced.

Internode distance

- intermediate phenotype: the 5^{th} internode below inflorescence is about 2.5 cm long and 9^{th} internode was about 0,5 cm long compared to 5 cm and 2 cm for the length of the 5^{th} and 9^{th} internode respectively, in WT plants.
- strong phenotype: plant *AtCKX1-50* The length of the 20^{th} internode from the bottom measured at day 131 after germination was 1.3 ± 0.4 mm compared to 39.2 ± 3.8 mm for WT

Apical dominance and branching

More side branches were formed indicating reduced apical dominance compared to WT plants during vegetative growth (see Figure 9). The side branches overgrew the main stem, reaching a height of 40-45 cm for intermediate *AtCKX1* expressors. Even secondary branches appeared. However, the buds were not completely released from apical dominance, i.e. lateral shoots did not really

continue to develop. The reduced apical dominance might be due to reduced auxin production by the smaller shoot apical meristem (see Example 10).

Reproductive development

The onset of flowering in *AtCKX1* transgenics was delayed, the number of flowers and the seed yield per capsule was reduced. The size of flowers was not altered in transgenic plants and the weight of the individual seeds was comparable to the weight of seeds from wild type plants. Data for two representative *AtCKX1* transgenics is summarized below:

A. Onset of flowering

Line	Wild-type	AtCKX1-48	AtCKX1-50
Flowering time (DAG)	106.2 ± 3.3	193.3 ± 4.3	191.8 ± 3.8

Experimental: Data collected for at least ten plants per line. The full elongation of the first flower was defined as onset of flowering. DAG = days after germination.

B. Number of seed capsules per plant

Line	Wild-type	AtCKX1-48	AtCKX1-50
Number of capsules	83.33 ± 5.13	2.00 ± 1.00	2.60 ± 1.67

Experimental: Number of seed capsules was determined at least from 5 different plants. Please note that these plants were grown under greenhouse conditions during winter time. This affects negatively the number of flowers that are formed, in particular in the transgenic clones. However, the general picture that they form a reduced number of flowers is correct. n.d., not determined

C. Seed yield / capsule (mg)

Line	Wild-type	AtCKX1-48	AtCKX1-50
Seed/capsule (mg)	87.41 ± 28.75	23.83 ± 13.36	61.8 ± 40.66

Experimental: Seed yield was determined for at least 12 seed capsules. The size of seed capsules was very variable, hence the large standard deviations. n.d., not determined

D. Weight of 100 seeds (mg)

Line	Wild-type	AtCKX1-48	AtCKX1-50
Seeds weight (mg)	9.73 ± 0.44	10.70 ± 1.60	9.54 ± 0.94

Experimental: *The seed biomass was determined as the weight of 100 seed from at least 5 different seed capsules. n.d., not determined*

3.2 In *Arabidopsis*

- onset of germination was same as for WT
- the total root system was enlarged and the number of side roots and adventitious roots was enhanced (see Figure 4 A through D)
- the growth of aerial organs was reduced resulting in a dwarfed phenotype (see Figure 4 E and F) and the leaf biomass was reduced. Leaf and flower formation is delayed.
- the life cycle was longer compared to WT and the seed yield was lower compared to WT

The following morphometric data illustrate these phenotypes:

Root development

A. Total length of the root system

Line	Wild-type	AtCKX1-11	AtCKX1-15
Length (mm)	32.5	76.5	68.4

B. Primary root length

Line	Wild-type	AtCKX1-11	AtCKX1-15
Length (mm)	32.3 ± 3.8	52.3 ± 4.8	39.9 ± 4.2

C. Lateral roots (LR) length

Line	Wild-type	AtCKX1-11	AtCKX1-15
Length (mm)	0.2 ± 0.4	15.6 ± 11.0	10.4 ± 7.6

D. Adventitious roots length

Line	Wild-type	AtCKX1-11	AtCKX1-15
Length (mm)	0.03 ± 0.18	8.6 ± 8.5	19.1 ± 11.0

E. Number of lateral roots (LR)

Line	Wild-type	AtCKX1-11	AtCKX1-15
Number of LR	0.3 ± 0.5	10.4 ± 5.4	2.6 ± 1.1

F. Number of adventitious roots (AR)

Line	Wild-type	AtCKX1-11	AtCKX1-15
Number of AR	0.03 ± 0.18	1.6 ± 1.1	2.6 ± 1.1

Experimental: Measurements were carried out on plants 8 days after germination in vitro on MS medium. At least 17 plants per line were scored.

Shoot development

A. Leaf surface

Line	Wild-type	AtCKX1-11-7 T3 homozygous plants	AtCKX1-11-12 T3 homozygous plants	AtCKX1-15-1 T3 homozygous plants
Leaf surface (cm^2)	21.16 ± 1.73	2.28 ± 0.58	2.62 ± 0.28	1.66 ± 0.22

Experimental: Leaf surface area of main rosette leaves formed after 30 days after germination was measured. 3 plants per clone were analyzed.

Reproductive development

Onset of flowering

Line	Wild-type	AtCKX1-11 T3 heterozygous plants	AtCKX2-2 T2 heterozygous plants	AtCKX2-5 T2 heterozygous plants
Flowering time (DAG)	43.6 ± 5.8	69.7 ± 9.4	51.2 ± 4.1	45.1 ± 6.9

Experimental: Plants were grown under greenhouse condition. At least 13 plants per clone were analyzed. DAG = days after germination

Conclusion: The analysis of *AtCKX1* transgenic *Arabidopsis* plants confirmed largely the results obtained from tobacco and indicates the general nature of the consequences of a reduced cytokinin content. The total root system was enlarged (the total root length was increased app. 110-140% in *AtCKX1* transgenics), the shoot developed more slowly (retarded flowering) and the leaf biomass was reduced. The seed yield was lower in the transgenics as well.

Example 4. Transgenic plants overexpressing *AtCKX2* showed increased cytokinin oxidase activity and altered plant morphology

1. Description of the cloning process

The following primers were used to PCR amplify the AtCKX2 gene from *Arabidopsis thaliana*, accession Columbia (non-homologous sequences used for cloning are in lower case):

Sequence of 5´ primer: gcggtaccAGAGAGAGAAACATAAACAAATGGC (SEQ ID NO:15)

Sequence of 3´ primer: gcggtaccCAATTTTACTTCCACCAAAATGC (SEQ ID NO:16)

A 3104-bp PCR fragment, amplified by these primers, was inserted in the KpnI site of pUC19. The insert was sequenced to check that no differences to the published sequence were introduced by the PCR procedure. The KpnI/KpnI fragment of this vector was subcloned in the KpnI site downstream of a modified CaMV 35S promoter (carrying three tetracycline operator sequences) in the binary vector pBinHyg-Tx (Gatz *et al.*, 1992). The resulting construct was introduced into tobacco and *Arabidopsis thaliana* through *Agrobacterium*-mediated transformation, using standard transformation protocols.

2. Molecular analysis of the transgenic lines

Several transgenic lines were identified that synthesize the *AtCKX2* transcript at high levels (Fig 6). Transgenic lines expressing *AtCKX2* transcript also showed

increased cytokinin oxidase activity. This is exemplified for 2 tobacco and 3 Arabidopsis lines in Table 7. This result proves that the *AtCKX2* gene encodes a protein with cytokinin oxidase activity.

Table 7. Cytokinin oxidase activity in *AtCKX2* transgenic plant tissues

Sample		
Plant species and tissue	**Plant line**	**Cytokinin oxidase activity (nmol Ade/mg protein.h)**
Arabidopsis callus	Col-0 wild-type	0.037
	CKX2-15	0.351
	CKX2-17	0.380
	CKX2-55	0.265
Tobacco leaves	SNN wild-type	0.009
	CKX2-SNN-18	0.091
	CKX2-SNN-19	0.091

3. Phenotypic description of the transgenic lines

<u>3.1 In tobacco (see Fig 7 to 10):</u>

Three categories of phenotype:

1) strong - 15 clones (similar to intermediate phenotype of *AtCKX1*)
2) weak - 6 clones
3) others - similar to WT plants, 7 clones

<u>Aerial plant parts</u>

The observations concerning plant height, internode distance, branching, leaf form and yellowing were similar as for *AtCKX1* transgenics with some generally minor quantitative differences in that the dwarfing characteristics were more severe in *AtCKX1* transgenics than in *AtCKX2* transgenics (compare *AtCKX1* plants with *AtCKX2* plants in Figure 7 A and B). This is illustrated below for stem elongation

and internode distance measurements of clones with a strong phenotype *AtCKX2-38* and *AtCKX2-40*:

Stem elongation

Line	Wild-type	AtCKX2-38	AtCKX2-40
Days after germination	Height (cm)	Height (cm)	Height (cm)
47	9.5 ± 0.5	2.4 ± 0.1	2.6 ± 0.2
58	22.4 ± 2.3	5.5 ± 0.7	5.3 ± 0.5
68	35.3 ± 2.6	7.1 ± 0.8	7.0 ± 0.7
100	113.3 ± 9.8	15.5 ± 2.5	20.3 ± 6.4
117	138.6 ± 8.1	19.8 ± 3.8	29.5 ± 6.0
131	139.0 ± 9.3	26.5 ± 7.0	33.4 ± 5.8
152	136.6 ± 10.4	33.7 ± 6.3	33.9 ± 6.4
165		36.2 ± 4.3	

Experimental: Plants were grown in soil in a green house. Data were collected from at least ten plants per line.

Internode distance

Line	Wild-type	AtCKX2-38
Internode distance (mm)	39.2 ± 3.8	7.2 ± 1.6

Experimental: The length of the 20th internode from the bottom was measured at day 131 after germination.

Roots

In vitro grown plants highly expressing the gene were easily distinguishable from WT plants by their ability to form more roots which are thicker (stronger) as well as by forming aerial roots along the stem.

The primary root was longer and the number of lateral and adventitious roots was higher as illustrated in Figure 8 C for *AtCKX2-38* overexpressing seedlings (see also Example 9).

The dose-response curve of root growth inhibition by exogenous cytokinin showed that roots of transgenic seedlings were more cytokinin resistant than WT roots (Figure 8 D). The resistance of *AtCKX1-28* transgenics to iPR was less

marked than for *AtCKX2-38*, which is consistent with the smaller changes in iP-type cytokinins in the latter (see Table 10).

An increase in fresh and dry weight of the root biomass of TO lines of AtCKX2 transgenic plants compared to WT was observed for plant grown in soil, as illustrated in the following table:

Line	Wild-type	AtCKX2 (T0)
Fresh weight (g)	45.2 ± 15.4	77.1 ± 21.3
Dry weight (g)	6.3 ± 1.9	8.6 ± 2.2

Experimental: Six WT plants and six independent T0 lines of 35S::AtCKX2 clone were grown on soil. After flowering the root system was washed with water, the soil was removed as far as possible and the fresh weight and dry weight was measured.

An increase in fresh and dry weight of the root biomass was also observed for F1 progeny of *AtCKX2* transgenics grown in hydroponics as compared to WT, as illustrated in the following table:

Line	Wild-type	AtCKX2-38	AtCKX2-40
Fresh weight ROOT (g)	19.76 ± 6.79	33.38 ± 7.76	50.04 ± 15.59
Dry weight ROOT (g)	2.36 ± 0.43	2.61 ± 0.39	3.52 ± 1.06
Fresh weight SHOOT (g)	159.8 ± 44.53	33.66 ± 2.67	48.84 ± 11.83
Fresh weight SHOOT/ROOT ratio	8.24 ± 0.63	1.04 ± 0.18	1.08 ± 0.51

Experimental: Soil grown plants were transferred 60 days after germination to a hydroponic system (Hoagland´s solution) and grown for additional 60 days. The hydroponic solution was aerated continuously and replaced by fresh solution every third day.

In summary, transgenic plants grown in hydroponic solution formed approximately 65-150% more root biomass (fresh weight) than wild type plants. The increase in dry weight was 10-50%. This difference is possibly in part due to the larger cell volume of the transgenics. This reduces the relative portion of cell walls, which forms the bulk of dry matter material. The shoot biomass was reduced to 20%-70% of wild type shoots. The difference in fresh weight leads to a shift in the shoot/root ratio, which was approximately 8 in wild type but approximately 1 in the transgenic clones.

Conclusion:

An increase in root growth and biomass was observed for *AtCKX2* transgenic seedlings and adult plants grown under different conditions compared to WT controls despite the fact that growth of the aerial plant parts is reduced. Quantitative differences were observed between different transgenic plants: higher increases in root biomass were observed for the strongest expressing clones.

Reproductive development

The onset of flowering in *AtCKX2* transgenics was delayed, the number of flowers and the seed yield per capsule was reduced. These effects were very similar to those observed in the *AtCKX1* transgenic plants but they were less prominent in the *AtCKX2* transgenics, as indicated in the tables below. The size of flowers was not altered in transgenic plants and the weight of the individual seeds was comparable to the weight of seeds from wild type plants.

A. Onset of flowering

Line	Wild-type	AtCKX1-48	AtCKX1-50	AtCKX2-38	AtCKX2-40
Flowering time (DAG)	106.2 ± 3.3	193.3 ± 4.3	191.8 ± 3.8	140.6 ± 6.5	121.9 ± 9.8

Experimental: Data collected for at least ten plants per line. The full elongation of the first flower was defined as onset of flowering. DAG = days after germination.

B. Number of seed capsules per plant

Line	Wild-type	AtCKX1-48	AtCKX1-50	AtCKX2-38	AtCKX2-40
Number of capsules	83.33 ± 5.13	2.00 ± 1.00	2.60 ± 1.67	4.30 ± 2.58	n.d.

Experimental: Number of seed capsules was determined at least from 5 different plants. Please note that these plants were grown under green house conditions during winter time. This affects negatively the number of flowers that are formed, in particular in the transgenic clones. However, the general picture that they form a reduced number of flowers is correct. n.d., not determined

C. Seed yield / capsule (mg)

Line	Wild-type	AtCKX1-48	AtCKX1-50	AtCKX2-38	AtCKX2-40
Seed/capsule (mg)	87.41 ± 28.75	23.83 ± 13.36	61.8 ± 40.66	46.98 ± 29.30	n.d.

Experimental: Seed yield was determined for at least 12 seed capsules. The size of seed capsules was very variable, hence the large standard deviations. n.d., not determined

D. Weight of 100 seeds (mg)

Line	Wild-type	AtCKX1-48	AtCKX1-50	AtCKX2-38	AtCKX2-40
Seeds weight (mg)	9.73 ± 0.44	10.70 ± 1.60	9.54 ± 0.94	10.16 ± 0.47	n.d.

Experimental: *The seed biomass was determined as the weight of 100 seed from at least 5 different seed capsules. n.d., not determined*

3.2 In Arabidopsis:

The following morphometric data were obtained for *AtCKX2* transgenics:

Root development

A. Total length of the root system

Line	Wild-type	AtCKX2-2	AtCKX2-5
Length (mm)	32.5	50.6	48.5

B. Primary root length

Line	Wild-type	AtCKX2-2	AtCKX2-5
Length (mm)	32.3 ± 3.8	30.7 ± 4.8	31.6 ± 6.8

C. Lateral roots length

Line	Wild-type	AtCKX2-2	AtCKX2-5
Length (mm)	0.2 ± 0.4	5.5 ± 9.0	1.9 ± 2.5

D. Adventitious roots length

Line	Wild-type	AtCKX2-2	AtCKX2-5
Length (mm)	0.03 ± 0.18	14.4 ± 10.2	14.9 ± 9.1

E. Number of lateral roots (LR)

Line	Wild-type	AtCKX2-2	AtCKX2-5
Number of LR	0.3 ± 0.5	2.9 ± 2.3	1.9 ± 1.0

F. Number of adventitious roots (AR)

Line	Wild-type	AtCKX2-2	AtCKX2-5
Number of AR	0.03 ± 0.18	1.8 ± 0.9	1.8 ± 1.0

Experimental: Measurements were carried out on plants 8 d.a.g. in vitro on MS medium. At least 17 plants per line were scored.

Shoot development

Leaf surface

Line	Wild-type	AtCKX2-2 T2 heterozygous plants	AtCKX2-5 T2 heterozygous plants	AtCKX2-9 T2 heterozygous plants
Leaf surface (cm^2)	21.16 ± 1.73	8.20 ± 2.35	8.22 ± 0.55	7.72 ± 0.85

Experimental: Leaf surface area of main rosette leaves formed after 30 days after germination was measured. 3 plants per clone were analyzed.

Reproductive development

Onset of flowering

Line	Wild-type	AtCKX1-11 T3 heterozygous plants	AtCKX2-2 T2 heterozygous plants	AtCKX2-5 T2 heterozygous plants
Flowering time (DAG)	43.6 ± 5.8	69.7 ± 9.4	51.2 ± 4.1	45.1 ± 6.9

Experimental: Plants were grown under greenhouse condition. At least 13 plants per clone were analyzed. DAG = days after germination.

Conclusion: Arabidopsis *AtCKX2* transgenics had reduced leaf biomass and a dwarfing phenotype similar to *AtCKX1* transgenics (compare Figure 5 with Figure 4 F). The total root system was also enlarged in *AtCKX2* transgenic Arabidopsis. The total root length is increased approximately 50% in *AtCKX2* transgenics. The *AtCKX1* transgenics have longer primary roots, more side roots and form more adventitious roots. *AtCKX2* transgenics lack the enhanced growth of the primary root but form more side roots and lateral roots than WT.

Summary:

The phenotypes observed for *AtCKX2* transgenics were very similar but not identical to the *AtCKX1* transgenics, which in turn were very similar but not identical to the results obtained for the tobacco transgenics. This confirms the general nature of the consequences of a reduced cytokinin content in these two plant species and therefore, similar phenotypes can be expected in other plant species as well. The main difference between tobacco and Arabidopsis is the lack of enhanced primary root growth in *AtCKX2* overexpressing plants.

Example 5. Transgenic plants overexpressing *AtCKX3* showed increased cytokinin oxidase activity and altered plant morphology

1. Description of the cloning process

The following primers were used to PCR amplify the *AtCKX3* gene from *Arabidopsis thaliana*, accession Columbia (non-homologous sequences used for cloning are in lower case):

Sequence of 5´ primer: gcggtaccTTCATTGATAAGAATCAAGCTATTCA (SEQ ID NO:17)

Sequence of 3´ primer: gcggtaccCAAAGTGGTGAGAACGACTAACA (SEQ ID NO:18)

A 3397-bp PCR fragment, produced by this PCR amplification, was inserted in the KpnI site of pBluescript. The insert was sequenced to confirm that the PCR product has no sequence changes as compared to the gene. The KpnI/KpnI fragment of this vector was subcloned in the KpnI site downstream of a modified CaMV 35S promoter (carrying three tetracycline operator sequences) in the binary vector pBinHyg-Tx (Gatz *et al.*, 1992). The resulting construct was introduced into tobacco and *Arabidopsis thaliana* through *Agrobacterium*-mediated transformation, using standard transformation protocols.

2. Molecular analysis of the transgenic lines

Several transgenic tobacco lines were identified that synthesize the *AtCKX3* transcript at high levels (Fig 11 A.). Transgenic tobacco lines expressing *AtCKX3* transcript also showed increased cytokinin oxidase activity. This is exemplified for three plants in Table 8. This proves that the *AtCKX3* gene encodes a protein with cytokinin oxidase activity.

Table 8. Cytokinin oxidase activity in *AtCKX4* transgenic plant tissues

Sample		
Plant species and tissue	**Plant line**	**Cytokinin oxidase activity (nmol Ade/mg protein.h)**
tobacco leaves	SNN wild-type	0.011
	CKX3-SNN-3	0.049
	CKX3-SNN-6	0.053
	CKX3-SNN-21	0.05

3. Plant phenotypic analysis

The phenotypes generated by overexpression of the *AtCKX3* gene in tobacco and *Arabidopsis* were basically similar as those of *AtCKX1* and *AtCKX2* expressing plants, i.e. enhanced rooting and dwarfing. However, overexpression of the *AtCKX3* gene in tobacco resulted in a stronger phenotype compared to *AtCKX2*. In this sense *AtCKX3* overexpression was more similar to *AtCKX1* overexpression.

<u>Example 6. Transgenic plants overexpressing *AtCKX4* showed increased cytokinin oxidase activity and altered plant morphology</u>

1. Description of the cloning process

The following primers were used to PCR amplify the AtCKX4 gene from *Arabidopsis thaliana*, accession Columbia (non-homologous sequences used for cloning are in lower case):

Sequence of 5´ primer: gcggtaccCCCATTAACCTACCCGTTTG (SEQ ID NO:19)

Sequence of 3´ primer: gcggtaccAGACGATGAACGTACTTGTCTGTA (SEQ ID NO:20)

A 2890-bp PCR fragment, produced by this PCR amplification, was inserted in the KpnI site of pBluescript. The insert was sequenced to confirm that the PCR product has no sequence changes as compared to the gene. The KpnI/KpnI fragment of this vector was subcloned in the KpnI site downstream of a modified CaMV 35S promoter (carrying three tetracycline operator sequences) in the binary vector pBinHyg-Tx (Gatz *et al.*, 1992). The resulting construct was introduced into tobacco and *Arabidopsis thaliana* through *Agrobacterium*-mediated transformation, using standard transformation protocols.

2. Molecular analysis of the transgenic lines

Several transgenic tobacco lines synthesized the *AtCKX4* transcript at high levels (Fig 11 B.). Transgenic lines expressing *AtCKX4* transcript also showed increased cytokinin oxidase activity. This is exemplified for 3 A*rabidopsis* and 3 tobacco lines in Table 9. This result proves that the *AtCKX4* gene encodes a protein with cytokinin oxidase activity.

Table 9. Cytokinin oxidase activity in *AtCKX4* transgenic plant tissues

Sample		
Plant species and tissue	**Plant line**	**Cytokinin oxidase activity (nmol Ade/mg protein.h)**
Arabidopsis callus	Col-0 wild-type	0.037
	CKX4-37	0.244
	CKX4-40	0.258
	CKX4-41	0.320
tobacco leaves	SNN wild-type	0.011
	CKX4-SNN-3	0.089
	CKX4-SNN-18	0.085
	CKX4-SNN-27	0.096

Overall, the data showed that the apparent K_m values for the four cytokinin oxidases were in the range of 0.2 to 9.5 μM with iP as substrate, which further

demonstrates that the proteins encoded by *AtCKX1* through *4* are indeed cytokinin oxidase enzymes as disclosed herein.

3. Plant phenotypic analysis

The phenotypes generated by overexpression of the *AtCKX4* gene in tobacco and *Arabidopsis* were basically similar as those of *AtCKX1* and *AtCKX2* expressing plants, i.e. enhanced rooting, reduced apical dominance, dwarfing and yellowing of intercostal regions in older leaves of tobacco. An additional phenotype in tobacco was lanceolate leaves (altered length-to-width ratio).

<u>General observations of *AtCKX* overexpressing tobacco plants</u>

Overall, the phenotypic analysis demonstrated that *AtCKX* gene overexpression caused drastic developmental alterations in the plant shoot and root system in tobacco, including enhanced development of the root system and dwarfing of the aerial plant part. Other effects such as altered leaf senescence, formation of adventitious root on stems, and others were also observed as disclosed herein. The alterations were very similar, but not identical, for the different genes. In tobacco, *AtCKX1* and *AtCKX3* overexpressors were alike as were *AtCKX2* and *AtCKX4*. Generally, the two former showed higher expression of the traits, particularly in the shoot. Therefore, a particular cytokinin oxidase gene may be preferred for achieving the phenotypes that are described in the embodiments of this invention.

<u>**Example 7. Cloning of the *AtCKX5* gene**</u>

The following primers were used to PCR amplify the AtCKX5 gene from *Arabidopsis thaliana*, accession Columbia (non-homologous sequences used for cloning are in lower case):

Sequence of 5´ primer: ggggtaccTTG<u>ATG</u>AATCGTGAA*ATG*AC (SEQ ID NO:21)

Sequence of 3´ primer: ggggtaccCTTTCCTCTTGGTTTTGTCCTGT (SEQ ID NO:22)

The sequence of the 5' primer includes the two potential start codons of the AtCKX5 protein, the most 5' start codon is underlined and a second ATG is indicated in italics.

A 2843-bp PCR fragment, produced by this PCR amplification, was inserted as a blunt-end product in pCR-Blunt II-TOPO cloning vector (Invitrogen).

Example 8. Cloning of the *AtCKX6* gene

The following primers were used to PCR amplify the AtCKX6 gene from *Arabidopsis thaliana*, accession Columbia (non-homologous sequences used for cloning are in lower case):

Sequence of 5´ primer: gctctagaTCAGGAAAAGAACCATGCTTATAG (SEQ ID NO:23)

Sequence of 3´ primer: gctctagaTCATGAGTATGAGACTGCCTTTTG (SEQ ID NO:24)

A 1949-bp PCR fragment, produced by this PCR amplification, was inserted as a blunt-end product in pCR-Blunt II-TOPO cloning vector (Invitrogen).

Example 9. Tobacco seedling growth test demonstrated early vigor of *AtCKX* transgenics

Seeds of *AtCKX1-50* and *AtCKX2-38* overexpressing transgenics and WT tobacco were sown *in vitro* on MS medium, brought to culture room 4 days after cold treatment and germinated after 6 days. Observations on seedling growth were made 10 days after germination (see also Figure 8C) and are summarized below. At least 20 individuals were scored per clone. Similar data have been obtained in two other experiments.

A. Total length of the root system

Line	Wild-type	AtCKX1-50	AtCKX2-38
Length (mm)	61.1	122.0	106.5

B. Primary root length

Line	Wild-type	AtCKX1-50	AtCKX2-38
Length (mm)	32.3 ± 2.6	50.8 ± 4.5	52.4 ± 4.8

C. Lateral roots length

Line	Wild-type	AtCKX1-50	AtCKX2-38
Length (mm)	9.8 ± 5.5	18.0 ± 8.1	13.0 ± 6.0

D. Adventitious roots length

Line	Wild-type	AtCKX1-50	AtCKX2-38
Length (mm)	19.0 ± 5.0	53.0 ± 12.0	42.0 ± 9.8

E. Number of lateral roots (LR)

Line	Wild-type	AtCKX1-50	AtCKX2-38
Number of LR	1.9 ± 0.9	6.5 ± 2.2	5.6 ± 2.0

F. Number of adventitious roots (AR)

Line	Wild-type	AtCKX1-50	AtCKX2-38
Number of AR	2.2 ± 0.6	3.5 ± 0.9	3.6 ± 1.3

AtCKX1 and *AtCKX2* plants, general observations:

Seedlings of *AtCKX1* and *AtCKX2* overexpressing tobacco plants had 60% more adventitious roots and three times more lateral roots than untransformed control plants 10 days after germination. The length of the primary root was increased by about 70%. This – together with more and longer side roots and secondary roots – resulted in a 70-100% increase in total root length. These results showed that overexpression of cytokinin oxidase enhances the growth and development of both the main root and the adventitious roots, resulting in early vigor.

Example 10. Histological analysis of altered plant morphology in *AtCKX1* overexpressing tobacco plants

Microscopic analysis of different tissues revealed that the morphological changes in *AtCKX* transgenics are reflected by distinct changes in cell number and rate of cell formation (see Figure 10). The shoot apical meristem (SAM) of *AtCKX1* transgenics was smaller than in wild type and fewer cells occupy the space between the central zone and the peripheral zone of lateral organ formation, but the cells were of the same size (Figure 10 A). The reduced cell number and size of the SAM as a consequence of a reduced cytokinin content indicates that cytokinins have a role in the control of SAM proliferation. No obvious changes in the differentiation pattern occurred, suggesting that the spatial organization of the differentiation zones in the SAM is largely independent from cell number and from the local cytokinin concentration. The overall tissue pattern of leaves in cytokinin oxidase overexpressors was unchanged. However, the size of the phloem and xylem was significantly reduced (Figure 10 B). By contrast, the average cell size of leaf parenchyma and epidermal cells was increased four- to fivefold (Figure 10 C, D). New cells of *AtCKX1* transgenics are formed at 3-4% of the rate of wild type leaves and final leaf cell number was estimated to be in the range of 5-6% of wild type. This indicates an absolute requirement for cytokinins in leaves to maintain the cell division cycle. Neither cell size nor cell form of floral organs was altered and seed yield per capsule was similar in wild type and *AtCKX* transgenic plants. The cell population of root meristems of *AtCKX1* transgenic plants was enlarged approximately 4-fold and the cell numbers in both the central and lateral columnella were enhanced (Figure 10 E, F). The final root diameter was increased by 60% due to an increased diameter of all types of root cells. The radial root patterns was identical in wild type and transgenics, with the exception that frequently a fourth layer of cortex cells was noted in transgenic roots (Figure 10 G). The increased cell number and the slightly reduced cell length indicates that the enhanced root growth is due to an increased number of cycling cells rather than increased cell growth. In the presence of lowered cytokinin content, root meristem cells must undergo additional rounds of mitosis before they leave the meristem and start to elongate. The exit from the meristem is therefore regulated by a mechanism that is sensitive to cytokinins. Apparently, cytokinins have a negative regulatory role in the root meristem and wild type cytokinin concentrations are inhibitory to the development of a maximal root

system. Therefore, reducing the level of active cytokinins by overexpressing cytokinin oxidases stimulates root development, which results in an increase in the size of the root with more lateral and adventitious roots as compared to WT plants.

Example 11. ***AtCKX1*** **and** ***AtCKX2*****- overexpressing tobacco plants had a reduced cytokinin content.**

Among the 16 different cytokinin metabolites that were measured, the greatest change occurred in the iP-type cytokinins in *AtCKX2* overexpressers (Table 10): the overall decrease in the content of iP-type cytokinins is more pronounced in *AtCKX2* expressing plants than in *AtCKX1* transgenics. *AtCKX1* transgenics showed a stronger phenotype in the shoot. It is not known which cytokinin metabolite is relevant for the different traits that were analysed. It may be that different cytokinin forms play different roles in the various development processes. Smaller alterations were noted for Z-type cytokinins, which could be due to a different accessibility of the substrate or a lower substrate specificity of the protein. The total content of iP and Z metabolites in individual transgenic clones was between 31% and 63% of wild type. The cytokinin reserve pool of *O*-glucosides was also lowered in the transgenics (Table 10). The concentration of *N*-glucosides and DHZ-type cytokinins was very low and was not or only marginally, altered in transgenic seedlings (data not shown).

Table 10. Cytokinin content of *AtCKX* transgenic plants. Cytokinin extraction, immunopurification, HPLC separation and quantification by ELISA methods was carried out as described by Faiss et al., 1997. Three independently pooled samples of approximately 100 two week old seedlings (2.5 g per sample) were analysed for each clone. Concentrations are in pmol x g fresh weight^{-1}. Abbreviations: iP, N^6-(Δ^2isopentenyl)adenine; iPR, N^6-(Δ^2isopentenyl)adenine riboside; iPRP, N^6-(Δ^2isopentenyl)adenine riboside 5′-monophosphate; Z, *trans*-zeatin; ZR, zeatin riboside; ZRP, zeatin riboside 5′-monophosphate; ZOG, zeatin *O*-glucoside; ZROG, zeatin riboside *O*-glucoside.

Line	WT	AtCKX1-2		AtCKX1-28		AtCKX2-38		AtCKX2-40	
Cytokinin meta-bolite	Concen-tration	Concen-tration	% of WT	Concen-tration	% of WT	Concen-tration	% of WT	Concen-tration	% of WT
iP	5.90 ± 1.80	4.76 ± 0.82	81	4.94 ± 2.62	84	1.82 ± 0.44	31	2.85 ± 0.62	48
iPR	2.36 ± 0.74	1.53 ± 0.14	65	0.75 ± 0.27	32	0.55 ± 0.39	23	0.89 ± 0.07	38
iPRP	-3.32 ± 0.73	0.87 ± 0.26	26	1.12 ± 0.13	34	0.80 ± 0.48	24	1.68 ± 0.45	51
Z	0.24 ± 0.06	0.17 ± 0.02	71	0.22 ± 0.03	92	0.21 ± 0.06	88	0.22 ± 0.02	92
ZR	0.60 ± 0.13	0.32 ± 0.12	53	0.34 ± 0.03	57	0.34 ± 0.15	57	0.32 ± 0.05	53
ZRP	0.39 ± 0.17	0.42 ± 0.11	107	0.28 ± 0.15	72	0.06 ± 0.01	15	0.17 ± 0.06	44
ZOG	0.46 ± 0.20	0.32 ± 0.09	70	0.26 ± 0.13	57	0.20 ± 0.07	43	0.12 ± 0.02	26
ZROG	0.48 ± 0.17	0.30 ± 0.06	63	0.47 ± 0.02	98	0.23 ± 0.05	48	0.30 ± 0.13	63
Total	13.75	8.69	63	8.38	61	4.21	31	6.55	48

Example 12. Grafting experiments showed that dwarfing and enhanced root development due to *AtCKX* overexpression is confined to transgenic tissues

To investigate which phenotypic effects of cytokinin oxidase overexpression are restricted to expressing tissues, i.e. are cell- or organ-autonomous traits, grafting experiments were performed. Reciprocal grafts were made between an *AtCKX2* transgenic tobacco plant and a WT tobacco. The transgenic plant used in this experiment was AtCKX2-38, which displayed a strong phenotype characterized by enhanced root growth and reduced development of the aerial plant parts. As

described in Example 3 through 6, these were two important phenotypes that resulted from cytokinin oxidase overexpression in tobacco and arabidopsis.

Plants were about 15 cm tall when grafted and the graft junction was about 10 cm above the soil. Figure 12 shows plants 15 weeks after grafting. The main results were that : (i) the aerial phenotype of a WT scion grafted on a transgenic rootstock was similar to the WT control graft (= WT scion on WT rootstock). Importantly, this showed that overexpression of the *AtCKX2* transgene in the rootstock did not induce dwarfing of the non-transgenic aerial parts of the plant (see Figure 12 A). Improved root growth of the transgenic rootstock was maintained, indicating that improved root growth of *AtCKX* transgenics is autonomous and does not depend on an *AtCKX* transgenic shoot (Figure 12 C). Interestingly, the WT scions grafted on the transgenic rootstocks looked healthier and were better developed. Notably, senescence of the basal leaves was retarded in these plants (see Figure 12 A); (ii) the transgenic scion grafted on the WT rootstock looked similar to the aerial part of the transgenic plant from which it was derived, i.e. the shoot dwarfing phenotype is also autonomous and not dependent on the improved root growth (see Figure 12 B).

In addition to the above-mentioned better appearance of WT shoots grafted on a transgenic rootstock, the formation of adventitious roots on the basal part of WT shoots was noted (Figure 12 D, right plant). Formation of adventitious roots also occurred on the stem of *AtCKX* transgenics but not on stems of WT control grafts (Figure 12 D, left plant) and therefore seems to be a non-autonomous trait.

In summary, it is disclosed in this invention that enhanced root formation and dwarfing of the shoot in *AtCKX* overexpressing tobacco are autonomous traits and can be uncoupled by grafting procedures. Surprisingly, grafting of a WT scion on an *AtCKX* transgenic rootstock resulted in more vigorously growing plants and retardation of leaf senescence.

As an alternative to grafting, tissue-specific promoters could be used for uncoupling the autonomous phenotypic effects of cytokinin overexpression. Therefore, it is disclosed in this invention that cytokinin oxidase overexpression in

a tissue specific manner can be used to alter the morphology of a plant such as the shoot or root system.

Example 13. Expression of an *AtCKX* gene under a root-specific promoter in transgenic plants leads to increased root production

An *AtCKX* gene (see example 4) is cloned under control of the root clavata homolog promoter of *Arabidopsis* (SEQ ID NO: 36) , which is a promoter that drives root-specific expression. Other root-specific promoters may also be used for the purpose of this invention. See Table 5 for exemplary root-specific promoters.

Transgenic plants expressing the *AtCKX* gene specifically in the roots show increased root production without negatively affecting growth and development of the aerial parts of the plant. Positive effects on leaf senescence and growth of aerial plant parts are observed.

Example 14. Suppression of an *AtCKX* gene under a senescence-induced promoter in transgenic plants leads to delayed leaf senescence and enhanced seed yield.

A chimeric gene construct derived from an *AtCKX* gene and designed to suppress expression of endogenous cytokinin oxidase gene(s) is cloned under control of a senescence-induced promoter. For example, promoters derived from senescence-associated genes (SAG) such as the SAG12 promoter can be used (Quirino et al., 2000). Transgenic plants suppressing endogenous cytokinin oxidase gene(s) specifically in senescing leaves show delayed leaf senescence and higher seed yield without negatively affecting the morphology and growth and development of the plant.

Example 15. Overexpression of an *AtCKX* gene in the female reproductive organs leads to parthenocarpic fruit development

The open reading frame of an *AtCKX* gene is cloned under control of a promoter that confers overexpression in the female reproductive organs such as for example the DefH9 promoter from *Antirrhinum majus* or one of its homologues, which

have high expression specificity in the placenta and ovules. Transgenic plants with enhanced cytokinin oxidase activity in these tissues show parthenocarpic fruit development.

Example 16. Overexpression of AtCKX genes result in increased seed and cotyledon size

Transgenic *Arabidopsis thaliana* plants that overexpress cytokinin oxidase (*AtCKX*) genes under control of the 35S promoter as described supra. Transgenic plants, in particular those expressing the *AtCKX1* and *AtCKX3* genes, developed seeds with increased size which was almost entirely due to an enlarged embryo. Details of the seed, embryo and early postembryonic phenotypes are shown in Figures 13 A through 13E. Table 11 shows seed weight of wild type and two independent clones for each of the four investigated AtCKX genes. Average weight was obtained by analysing five different batches of 200 seeds for each clone. A quantitative evaluation showed that the seed weight of *AtCKX1* and *AtCKX3* expressing clones was app. 1.8-2.3-fold higher than in wild type. Gain of weight for seeds of *AtCKX2* and *AtCKX4* expressing lines was in the range of 10-25% (Table 11 and Fig. 14).

The increases in size and weight for seeds, embryos, and cotyledons are unexpected as a reduced cytokinin content would have been expected to be associated with a reduced organ growth. One possible reason for the increases in seed, embryo, and cotyledon size is a previously unknown negative regulatory function of cytokinins in these storage organs. A negative regulatory functions of cytokinins in the control of organ growth is so far only known from roots (Werner et al. 2001). We propose, therefore, that localized expression of cytokinin oxidase genes in tissues where growth is negatively regulated by cytokinins leads to enhanced growth of this tissue. For example, localized expression of *CKX* genes during cotyledon development likely leads to enhanced growth of cotyledons and in species with cotyledons as storage organs, to enhanced yield and to an enhanced growth performance of seedlings. Total number of seeds is lowered in *AtCKX1* and *AtCKX3* expressers. There have been no previous reports however, of lower seed number in *Arabidopsis* being linked to an increase in size.

TABLE 11

	WT	CKX1-11-7	CKX1-15-1	CKX2-2-4	CKX2-9-3	CKX3-9-4	CKX3-12-13	CKX4-37-2	CKX4-41-7
Seed Weight	0.0158±0.0009	0.0372±0.0015	0.0352±0.0023	0.0201±0.0017	0.0180±0.0001	0.0340±0.0027	0.0280±0.0027	0.0185±0.0004	0.0179±0.0007
% of WT	100	235.5	222.6	126.7	113.7	215.0	176.7	116.8	112.7

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